10-27-2016 12:00 AM

The Role of Cholinergic Neurotransmission in Sensory Filtering and Sensorimotor Gating

Erin Azzopardi
The University of Western Ontario

Supervisor
Dr. Susanne Schmid
The University of Western Ontario

Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

Abstract

At every moment, our brain is bombarded with sensory information. How we filter and process sensory information is critical for daily functioning and cognition. Examples of sensory filtering include habituation (a progressive decrease in responding) and prepulse inhibition (PPI, gating of responding). Our aim is to understand the differential role acetylcholine (ACh) plays in these processes.

To study this we used both reflexive (acoustic startle response: ASR) and non-reflexive (locomotor) behaviours. PPI is hypothesized to occur via inhibitory cholinergic projections from the Pedunculopontine Tegmental Nucleus (PPT) to the startle pathway. The role of ACh in habituation of reflexive and non-reflexive behaviours is controversial. We found that, contrary to the predictions of the field, ACh modulated, not mediated, PPI. There was no impairment of PPI in cholinergic deficient mice. When we inhibited PPT cholinergic neurons using DREADDs we did not detect an impairment of PPI. Likewise, we were unable to induce PPI by optogenetic activation of these neurons.

Instead we found that cholinergic function is critical for long-term habituation (decrement occurring across days) as cholinergic deficient mice showed an impairment which was rescued by galantamine. Furthermore, inhibition of PPT cholinergic cells decreased startle magnitude, whereas optogenetic activation of cholinergic PPT neurons increased startle. This demonstrates that these neurons are critical for regulating startle reactivity. Despite modulating reflexive behaviours, PPT cholinergic inhibition did not impact habituation of locomotion, re-affirming differential regulation of habituation of reflexive and non-reflexive behaviours.

To uncover which cholinergic receptor type mediates effects on PPI and habituation we used an α7-nicotinic acetylcholine receptor (nAChR) knock-out mouse. These mice displayed a mild impairment of PPI, and no enhancement of startle magnitude or PPI via nicotine. This suggests ACh modulates PPI through this receptor, and confirms that cholinergic function enhances startle. Of interest, optogenetic enhancement of startle was blocked by nAChR antagonism.

In conclusion, we demonstrate that ACh modulates PPI through α7-nAChRs and that ACh is critical for regulating startle reactivity, indicating a potential role in long-term habituation or sensitization of startle. In contrast to the common view, cholinergic PPT function does not inhibit startle, ruling out a mechanistic role in PPI.

Keywords: Sensory Filtering, Sensorimotor Gating, Acoustic Startle Response, Habituation, Prepulse Inhibition, Acetylcholine, Pedunculopontine Tegmental Nucleus, Nicotinic Receptors, α7 Nicotinic Receptors, Locomotion, Sensitization, DREADDs, Optogenetics

Co-Authorship Statement

Chapter 1 was written by Erin Azzopardi with input from Dr. Susanne Schmid. Chapter 2, entitled “VAChT KD mice show normal prepulse inhibition but disrupted long-term habituation” was co-authored by Erin Azzopardi and Dr. Susanne Schmid. Erin performed all behavioural experiments, conducted data and statistical analysis, and provided intellectual input. Additional input was provided by Dr. Vania Prado, Dr. Marco Prado, and Dr. Xavier De Jaeger. Chapter 3, entitled “Sensorimotor gating and spatial learning in α7-nicotinic receptor knockout mice” was written by Erin Azzopardi with input from Dr. Susanne Schmid and Dr. Marei Typlt. Bryan Jenkins assisted with some behavioural experiments. Chapter 4, entitled “Cholinergic midbrain neurons modulate startle magnitude, but not PPI” was written by Erin Azzopardi with input from Dr. Susanne Schmid (exceptions indicated below). All behavioural experiments, data and statistical experiments were performed by Erin. Andrea Louttit assisted with some behaviour experiments in section 4.3.1. *In vitro* patch clamp recordings were performed solely by Dr. Tariq Zaman, and he provided input for section 4.3.2.5. *In vivo* electrophysiological recordings were performed in collaboration with Dr. Brian Allman and Ashley Schormans. Offline-analysis and spike sorting was performed solely by Ashley Schormans, but statistical analysis and figures (except A.2) were made by Erin Azzopardi. Ashley Schormans also provided substantial input to section 4.3.2.6. Dr. Cleusa De Oliveira assisted with the immunohistochemistry in section 4.2.3.4, but all imaging and data analysis was performed by Erin Azzopardi. Chapter 5 was written by Erin Azzopardi with input from Dr. Susanne Schmid.

Acknowledgements

Firstly, I'd like to thank my Supervisor, Dr. Susanne Schmid for all her guidance and support. Susanne was an excellent mentor and scientific role model. Her passion for science and dedication to her work is inspiring. Most importantly, I want to thank Susanne for making science fun, for never being afraid of negative results (which somehow I always managed to find) and having the vision to turn them into "positive" ones.

I'd like to thank all past and present members of the Schmid lab. Working in this lab was an awesome experience, and I could not have succeeded without all of my labmates. Although I wish I could name them all, I'd like to highlight Dr. Marei Typlt, Dr. Cleusa De Oliveira, Komal Shaikh, Mahabba Smoka, Bridget Valsamis, Tyler Brown, Ashley Schormans, and Andy Louttit for their friendship, assistance, and coffee breaks.

To my advisory committee, Dr. Vania Prado, Dr. Arthur Brown and Dr. Steve Laviolette I'd like express my appreciation for thoughtful and constructive feedback they provided throughout my PhD. Additionally, I'd like to thank Dr. Brian Allman for his help and guidance. He truly went above and beyond in order to support my graduate education.

Lastly I'd like to thank my parents for instilling in me a love of learning, for supporting me and being excellent examples of hard work, dedication, and kindness. Words cannot express how grateful I am to have parents like you. Without the love and encouragement of my family and friends I would certainly not stand where I am today. This is particularly true for Devin, who bore the grunt of the stress of thesis writing and somehow managed to keep me sane.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

ABC: Avidin Biotin Complex
ACh: Acetylcholine
ACSF: Artificial Cerebrospinal Fluid
ANOVA: Analysis of Variance
ASR: Acoustic Startle Response
AVG: Average
BAC: Bacterial Artificial Chromosome
BSA: Bovine Serum Albumin
CCAC: Canadian Council on Animal Care
ChT: Choline Transporter
ChAT: Choline Acetyltransferase
ChR2(H134R): Channel Rhodopsin 2
CNO: Clozapine N-Oxide
CPP: Conditioned Place Preference
DAB: 3,3'-Diaminobenzidine Tetrahydrochloride
dB SPL: Decibel Sound Pressure Level
DMSO: Dimethyl Sulfoxide
DREADDs: Designer Receptors Exclusively Activated by Designer Drugs
GABA: Gamma-aminobutyric acid
Gal: Galantamine
F: Female
IC: Inferior Colliculus
ICC: Intra-class Correlation
I/O: Input/Output
IP: Intraperitoneal
ISI: Interstimulus Interval
ITI: Intertrial Interval
LED: Light Emitting Diode
LDT: Laterodorsal Tegmental Nucleus
LTH: Long-term Habituation
M: Male
MEC: Mecamylamine
NA: Numerical Aperture
nAChR: Nicotinic Acetylcholine Receptor
NADPh: Nicotinamide adenine dinucleotide phosphate
PB: Phosphate Buffer
PCR: Polymerase Chain Reaction

PnC: Caudal Pontine Reticular Nucleus
PGB: Parabigeminal Nucleus
PPI: Prepulse Inhibition
PPT: Pedunculopontine Tegmental Nucleus
REM: Rapid Eye Movement
SAL: Saline
SC: Superior Colliculus
STH: Short-term Habituation
VAChT: Vesicular Acetylcholine Transporter
WT: Wild-type
YFP: Yellow Fluorescent Protein

1. Chapter 1: General Introduction

1.1 Modulations of the Acoustic Startle Response as a Tool to Study Information Processing Within the Brain

We live in a complex sensory world. The ability to filter sensory information is critical for accurate sensory information processing. In most instances, the filtering of sensory information is pre-attentive and can occur on different levels within sensory pathways. The ability to reduce the brain's awareness and behavioural responding to unnecessary sensory information is critical for proper daily functioning. Several psychiatric syndromes, including Schizophrenia and Autism Spectrum Disorders, are associated with disrupted sensory filtering. Sensory filtering can be analyzed in many different ways. However, in rodents and humans a commonly used measure is the acoustic startle response (ASR).

The ASR, as will be discussed in more detail in the following section, is a plastic response. It is subject to both enhancement, e.g. sensitization or prepulse facilitation, or attenuation, e.g. habituation and prepulse inhibition (PPI). Briefly, habituation is defined as the gradual decrease in startle magnitude to a startling stimulus after repeated exposure. This is an example of sensory filtering, which is thought to reduce the cognitive load of redundant sensory information so that the brain has more available resources to exert elsewhere. Prepulse Inhibition also reflects a decrease in startle; however it is due to the presence of a pre-pulse prior to the startling stimulus. PPI can be thought of as a gate-keeper, preventing irrelevant information from conscious awareness and thus precluding useless attentional and energy expenditure; it is often referred to as sensorimotor gating. Many studies in both animals and humans use habituation and PPI of the ASR as measures of sensory filtering and/or sensorimotor gating.

1.1.1 How We Process Sensory Information is Critical for Higher Order Cognitive Function

Sensory filtering and sensorimotor gating are pre-attentive processes. This type of basic cognition can provide the building blocks for higher-order cognitive function. By reducing the cognitive load of unnecessary sensory information, the brain has more available resources to exert elsewhere. On the other hand, PPI and habituation may be a proxy measure for the speed or efficiency of information processing within the brain. Evidence for this idea stems from studies that have found that sensory filtering and sensorimotor gating mechanisms correlate with higher cognitive function. For example, PPI efficacy has been correlated with spatial working memory in rodents (Singer et al., 2013; Oliveras et al., 2015). Additionally, rats selectively bred for low PPI showed increased perseverative errors across spatial and operant conditioning tasks (Freudenberg et al., 2007).

Many psychiatric illnesses display sensory filtering and PPI deficits (see section 1.1.2 for more detail), and within these disorders the severity of PPI or habituation deficits can correlate with cognitive functions across a variety of tasks. For example, in Schizophrenic patients, PPI deficits correlate with performance on the Global Assessment of Functioning Scale (Swerdlow et al., 2006) and severity of symptoms as assessed by the 18-item Brief Psychiatric Rating Scale score (Hazlett et al., 2007).

As sensory filtering and sensorimotor gating seem to be indicative of higher cognitive function, understanding the mechanisms underlying these processes may provide principles that could apply to the understanding of higher cognitive function and their disruptions as well.

1.1.2 Habituation and PPI Deficits are Present in a Number of Psychiatric Illnesses

A major behavioural hallmark of Schizophrenia is a deficit in sensorimotor gating as assessed by a disruption in PPI (Braff and Geyer, 1990; Parwani et al., 2000; Braff et al., 2001; Wynn et al., 2007; Takahashi et al., 2008). This deficit in inhibitory gating has been proposed to be linked to the hyper-vigilance displayed by these patients, suggesting that they over-process information within their environment (Freedman et al., 1994). PPI deficits are also present in a number of other psychiatric illnesses including Obsessive Compulsive Disorder (OCD), Tourette's Syndrome, Anxiety Disorders, and Huntington's Disorder (as reviewed by Braff et al., 2001; Geyer, 2006).

Impairments of PPI are thought reflect fundamental aspects of inhibitory processing that are differentially impacted in a variety of illnesses. For example, deficits of PPI in OCD and Schizophrenia may reflect a deficit in the gating of sensory and cognitive information whereas Tourette's and Huntington's reflect a reduced ability to gate motor responses. Whether this reflects co-morbidity or diagnostic overlap of impaired global inhibitory processes is unclear (Geyer, 2006).

Impairments of habituation of the startle response has been shown in animal models of Fragile X Syndrome (Nielsen et al., 2002), and Autism Spectrum Disorders (Ornitz et al., 1993) (however see Perry et al., 2007). Schizophrenic patients display impairments of both habituation and PPI of the startle response (Braff et al., 1992; Ludewig et al., 2002; Ludewig et al., 2003); whereas people suffering from OCD have normal habituation but impaired PPI (Hoenig et al., 2005). Impairments of habituation versus PPI may reflect subtle differences in cognitive deficits, but what these might be has not been extensively examined. Regardless, understanding the

mechanisms underlying both habituation and PPI may help improve treatment of these disorders and provide new drug targets.

1.1.3 The Acoustic Startle Response is an Excellent Tool to Investigate Information Processing

As mentioned, we use the ASR as a tool to study both habituation and PPI. The startle response is highly conserved across evolution. It occurs across species from invertebrates to humans. It is a multi-modal response and can be induced by visual, tactile, vestibular, and or auditory stimuli (Davis, 1984; Koch, 1999). Any sudden acoustic stimulus louder than 80 dB can elicit the ASR (Koch, 1999). This reflex commences with closure of the eyelids, followed by muscle contractions in the face, neck and skeletal muscles (Koch and Schnitzler, 1997; Koch, 1999). This is also accompanied by acceleration in heart rate and an arrest of ongoing behaviour (Gogan, 1970).

The ASR is highly adaptive, and it is an instinctive behaviour. Pilz and Schnitzler (1996) suggested that startling to an intense stimulus allows an organism to orient to potentially threatening stimuli and assess behavioural output accordingly. As startling halts ongoing behaviour, induces stiffening of neck musculature, limb flexion, and eyelid closure, it prepares the organism for a fight or flight response and protects vital organs in case of attack or injury. The ASR is an innate behaviour and requires no learning. It is functional as soon as the auditory system has been developed, which in rodents is as early as postnatal day 12 (Sheets et al., 1988; Kungel et al., 1996). The simplicity of the ASR pathway is fitting with the very short motor response latency (5-10 ms) observed following the onset of acoustic stimulation.

The primary startle pathway is well described. The sound is transduced into neuronal signals by hair cells of the inner ear which are innervated by spiral ganglion cells that project to the cochlear root (in rodents) and cochlear nucleus. The cochlear root neuron sends direct glutamatergic sensory afferents to the caudal pontine reticular nucleus (PnC), where they synapse on giant neurons. The giant neurons in turn directly synapse on facial, cranial and motoneurons in the spinal cord which when activated elicit a motor response (Davis et al., 1982a; Davis et al., 1982b; Koch et al., 1992; Lingenhohl and Friauf, 1992; Koch and Schnitzler, 1997). For schematic representation of this pathway please refer to figure 1.1.

This circuitry highlights the PnC as the sensorimotor interface of the ASR (Lingenhohl and Friauf, 1994). This area consolidates sensory information and transforms it into an adaptive behavioural output. The PnC does not only receive sensory projections, however. It also receives afferents from higher brain areas which can serve to modulate the startle response, making the ASR a plastic response (for review see Koch, 1999). As discussed previously, the ASR is subject to both enhancement and attenuation.

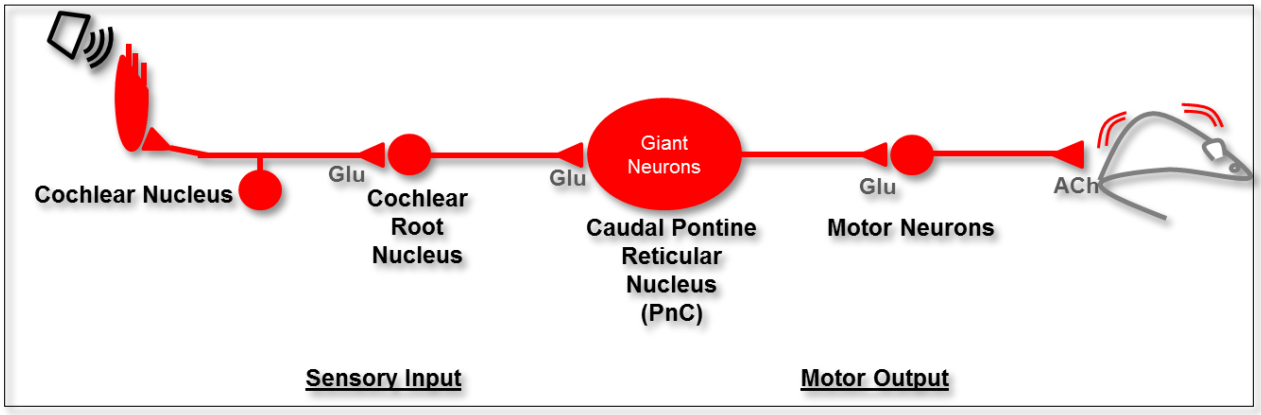

Figure 1.1 The Primary Startle Pathway

When a sound is detected by the ear it activates the auditory pathway. If the sound is sufficiently loud, this will cause excitation of the giant neurons in the PnC, which directly

innervate motor neurons of the spinal cord, eliciting a startle response. Adapted from (Simons-Weidenmaier et al., 2006).

1.2 An Introduction to Sensory Filtering: Habituation

Habituation is a modulation of information processing and is a form of non-associative learning. It refers to the progressive decrease of a behavioural response following repeated exposure to the behaviour-inducing stimuli. Virtually any behaviour can be subject to habituation. This review will focus on habituation of reflexes, like the ASR, and habituation of motivated behaviors, like locomotion.

Habituation of the ASR is denoted by the decrease in startle amplitude after repeated presentation of a loud, intense sound. In some organisms, such as *C. Elegans* it is a decrease in startle probability and not magnitude. In mammals, this decrement is most commonly negatively exponential and eventually, with continued exposure, reaches an asymptotic level of stable response magnitude (Rankin et al., 2009). If the stimulus is re-introduced after a prolonged period of absence, the organism will respond highly again, but this response will not be quite as great when it was presented for the first time (Thompson and Spencer, 1966).

This process of habituation is considered an adaptive form of learning. It is separated from simple muscle or receptor fatigue by displaying 10 core characteristics (for review see Rankin et al., 2009). Most critically, these include that habituation is stimulus specific, subject to spontaneous recovery, and lasts longer when it is induced by lower frequency stimulation (Best et al., 2005; Rankin, 2009). Habituation is adaptive as the unconditioned stimulus, the loud noise, contains no biologically relevant information. By lessening responding, the organism prevents useless expenditure of energy and attention (Koch, 1999).

1.2.1 There are Two Types of Habituation: Short and Long-Term

There are two forms of habituation, short-term and long-term. Short-term habituation refers to the decrease in startle magnitude that occurs when a loud sound is presented repeatedly within a short time span. Typically in a lab setting, short-term habituation occurs across trials within a single testing session. However, long-term habituation refers to the attenuation of startle that occurs across multiple testing sessions, or days.

A debate exists about whether short-term and long-term habituation are mediated by the same processes. Traditionally, it was thought that short-term habituation is non-associative, whereas long-term habituation was an associative learning process that involves retrieval mechanisms (Davis, 1970; Wagner, 1981; Sanderson and Bannerman, 2011). However, recent studies have suggested that long-term habituation is also non-associative (Pilz et al., 2014).

Despite both being non-associative, the neuronal underpinnings of these processes are very different. The mechanism underlying short-term habituation occurs directly within the startle pathway. It is largely hypothesized to be due to presynaptic depression, mediated by BK channels, between the secondary auditory afferent neurons and the giant neurons of the PnC (Weber et al., 2002; Simons-Weidenmaier et al., 2006; Typlt et al., 2013).

Much less is known about the mediation of long-term habituation of startle. Studies have implicated multiple brain structures. De-cerebrated rats show intact short-term but severely disrupted long-term habituation, providing evidence for involvement of the cortex (Leaton et al., 1985). While this was re-affirmed by Groves et al. (1974), they also noted that lesions to the posterior aspect of the mesencephalic reticular formation also resulted in an absence of long-term habituation. However, this was accompanied by a large reduction in startle magnitude as

well, making interpretation of the results difficult. Other studies demonstrated that the cerebellar vermis was critical (Timmann et al., 1998), as lesions to this area cause disruptions in long-term habituation (Leaton and Supple, 1986). Clearly, a greater understanding of the mechanisms and structures underlying long-term habituation is needed.

1.2.2 Habituation and Sensitization: The Dual Process Theory

The most prominent theory of habituation was proposed by Groves and Thompson (1970) termed the dual process theory. Although this theory is applicable to the habituation of all behaviours, for ease it will be discussed using habituation of the ASR as an example. Groves and Thompson suggested that following repeated exposure to a stimulus, behavioural outcome is dependent on two opposing processes: habituation and sensitization. Behaviourally, sensitization is the opposite of habituation, an enhancement of response magnitude (Koch, 1999).

Groves and Thompson hypothesized that habituation occurs within the stimulus-response pathway (which in this case would be the primary startle pathway), and that sensitization occurs in a separate "state" pathway (see also Poon and Schmid, 2012). Repeated exposure to a startling stimulus simultaneously activates both sensitization and habituation pathways. The input of these pathways is integrated at some point of the startle pathway, a likely candidate would be the PnC (Lingenhohl and Friauf, 1994), and the behavioural output equals the summative activity of these opposing processes.

The balance between sensitization and habituation is not only detectable on the resulting startle amplitude. The onset latency of the startle response itself is thought to reflect a state of

sensitization. In general, startle amplitude is negatively correlated with startle latency. However, in studies with testing parameters promoting habituation, startle latency decreases with startle amplitude. This is thought to be a reflection of a parallel increase in sensitization that impacts latency rather than the startle amplitude (Pilz and Schnitzler, 1996).

The neuronal underpinnings of the pathway of startle sensitization have not been well established. Furthermore, within the literature it is hard to distinguish if any manipulations that result in an increased startle reactivity in general can be classified as a mechanism of sensitization. Fear-potentiation of startle, where an aversive-conditioned stimulus is presented before a startling sound, has been well studied, but it is unclear if these mechanisms reflect the same type of plasticity required for general (non-associative) sensitization of the startle response. Regardless, sensitization of the startle response can be induced with electrical stimulation of the amygdala, an effect that was most reliably induced when stimulation sites targeted the ventral amygdalofugal pathway, which projects to lower brainstem nuclei (Rosen and Davis, 1988).

Although the amygdala has direct projections to the startle-mediating PnC, it appears that startle sensitization is relayed through intermediary nuclei. Potential relay sites include the periaqueductal grey area, substania nigra, deep mesencephalic nucleus, laterodorsal tegmental (LDT) or pedunculopontine tegmental nucleus (PPT; Hitchcock and Davis, 1986; Yeomans and Pollard, 1993; Fendt et al., 1994; Krase et al., 1994; Frankland et al., 1995; Frankland and Yeomans, 1995). Interestingly, electrical stimulation at certain potential relay centers (e.g. the PPT) can induce startle-like responses (Yeomans and Pollard, 1993).

The neuropeptide Substance P has also been strongly linked to unconditioned sensitization of startle (Krase et al., 1994). Interestingly, Kungel et al. (1994) found that Substance P innervation of the PnC mainly came from the PPT and LDT, and not the amygdala. They also observed that Substance P's ability to increase excitability of PnC neurons was increased with the co-administration of a cholinergic agonist. A subset of cholinergic midbrain neurons, including those in the PPT, are known to co-express Substance P markers (Standaert et al., 1986). Additionally, a rat line with hypocholinergia (Flinders Resistant Line) showed a decreased startle response magnitude, as well as sensitization of startle to tones (Markou et al., 1994). Taken together, this may suggest that cholinergic PPT function could be involved in sensitization of the startle response. However, a more widely accepted view of cholinergic-midbrain function has been that these neurons inhibit, or decrease, startle and that this is a mechanism for prepulse inhibition, as discussed in section 1.3.4 and 1.4.3.

1.2.3 Comparing Habituation of Reflexive vs. Non-Reflexive Behaviours

Previously, we discussed habituation in terms of the ASR, but habituation is not limited to just reflexive behaviours: it is applicable to all behaviours. Incremental attenuation of non-reflexive behaviours, in the literature referred to as emitted or motivated behaviours, are also important processes to understand. Research on habituation of non-reflexive behaviours can focus on everything from habituation of food foraging, aggression, to exploratory behaviour. Here, we focus on the latter, which is reflected in the habituation of locomotion in a new environment.

Habituation of locomotor behaviour generally displays the same features as habituation of reflexive behaviours. Both behaviours show short-term (intra-session) and long-term (inter-session) habituation. Overall, the principles discussed in section 1.2.1 can also extend to emitted behaviours. The most important shared feature is that habituation of non-reflexive behaviours is also not due to muscle or receptor fatigue (Rankin et al., 2009). Additionally, Muller and colleagues (1994) also defined decreases of locomotion within a testing session to reflect adaptation and decreases across sessions to reflect both adaptation and a memory for the environment. Groves and Thompson's dual process theory of habituation is also applicable to locomotor behaviour as Welker (1957) found that the motivational state of an animal can greatly alter habituation of locomotion. Therefore, both reflexive and non-reflexive habituation is subject to the same behavioural principles; however the underlying physiological mechanisms are thought to be very different.

This idea is best illustrated in a study by Williams, Hamilton and Carlton (1975). They examined both habituation of the ASR and locomotion. They observed that the two processes were ontogenetically dissociable. Habituation of locomotion differs according to age. Habituation of locomotion was not present in rodents younger than 13 days, but was present in older rodents, whereas habituation of the startle response was present as early as 13 days of age. This suggested that the two processes are actually mediated by distinct physiological mechanisms.

1.2.4 Habituation of the ASR and Acetylcholine

One of the first general theories of habituation was proposed by Carlton (1968). This theory stated that acetylcholine (ACh) was the neurotransmitter which mediated all inhibitory

processes, including habituation. Later studies, however, have greatly refuted this idea. There has been little evidence to suggest that ACh has a role in short term habituation of the ASR. In fact, most research has concluded that ACh is not involved in habituation of reflexes (Hughes, 1984). Across a variety of species, and developmental stages, anticholinergics have had no effect of the short-term habituation of the startle response (Williams et al., 1975; Brown, 1976).

Interestingly, none of these studies investigated long-term habituation. This is surprising as ACh innervates the entire brain, and is well poised to regulate a global process like long-term habituation. In part this could be because chronic manipulation of the cholinergic system was not available at the time these studies were performed, and it is likely that studying long-term habituation would require chronic rather than transient alterations. The cerebellar vermis, one of the structures linked to long-term habituation does receive cholinergic input from the lateral reticular nuclei (Barmack et al., 1992a; Barmack et al., 1992b). Furthermore, the mesencephalic reticular formation that has also been linked to long-term habituation, houses two cholinergic cell groups, the LDT and PPT. Despite this, no current study has looked at long-term habituation of the startle response and the role of ACh. This will be one of the goals of this thesis.

1.2.5 Habituation of Locomotor Behaviour and Acetylcholine

As previously discussed, there is little evidence to suggest that ACh is involved in habituation of the ASR, but there is much more evidence to suggest that ACh is very important for habituation of locomotor behaviour. The differential role that ACh plays in the habituation of locomotor behaviour compared to the ASR once again reiterates that the primary physiological

mechanisms of underlying these processes are distinct, as evidenced by different ontogenetic and cholinergic regulation.

There are many studies that have sought to understand the role of cholinergic function in habituation of locomotion. General septal cholinergic lesions by 192 IgG-saporin produced deficits in short-term habituation, but it also produced a decrease in overall locomotor behaviour compared to controls (Lamprea et al., 2003). Other studies have used *in vivo* microdialysis to monitor the profile of ACh release during open field tasks. In the hippocampus, it was observed that when rats are exposed to novel environments, extracellular ACh levels increased and that this is correlated with increased locomotor and exploratory behaviour (Ikegami, 1994; Thiel et al., 1998; Giovannini et al., 2001). This could suggest that ACh is important for encoding memories about new environments, or that it is somehow inducing increased motor activity. Theil and colleagues (1998) provide evidence against the later argument. They re-exposed animals to the same environment the following day and found that ACh levels increased again, but that locomotor behaviour decreased compared to the first exposure. In summary, evidence suggests that ACh has a role in the process of both short- and long-term habituation of locomotion whether through memory formation or arousal. Regardless, the cellular mechanism(s) and the potential cholinergic receptor subtypes involved remain elusive.

1.2.5 Habituation of Locomotor Behaviour and Cholinergic Receptors

ACh binds to two distinct receptor types: nicotinic and muscarinic receptors. Muscarinic receptors are metabotropic; there are 5 types of muscarinic receptors (M1-M5) all of which are G-protein coupled receptors. Activation of M1, M3, and M5 leads to decreased K^+ conductance

thereby increasing neuronal excitability, whereas M2 and M4 activation leads to increased K^+ and decreased Ca^+ conductance causing inhibition.

Green and Summerfield (1977) provided the first evidence of muscarinic receptor involvement in habituation of locomotor behaviour. They administered the muscarinic antagonist scopolamine and found that both short-term and long-term habituation were disrupted (Ukai et al., 1994; Brodkin, 1999).

More recently Schildein and colleagues (2002) investigated the role of nicotinic receptors in open field habituation. They found that local infusions of nicotine into the nucleus accumbens, right after exposure to an open field task, improved long-term habituation. Fittingly, they also observed that a general nicotinic antagonist, mecamylamine, disrupted this. If nicotine was given 5 hours after exposure to the open field, then again, they found disruptions in long-term habituation. This suggested that nicotinic receptors play an important, time dependant role in early consolidation phases of long-term habituation. While this provides an interesting basis for the role of the nucleus accumbens nicotinic receptors in habituation learning, questions remain about what specific nicotinic receptors are involved, and if there is a role for these receptors outside of the nucleus accumbens in short-term habituation of locomotor or other behaviours, as well as in the encoding processes of long-term habituation. Thus one of the goals of this thesis will be to understand what role the α7-nAChR specifically is playing in short and long-term habituation of locomotor behaviour and of the ASR.

1.3 An Introduction to Sensorimotor Gating: Prepulse Inhibition

Prepulse inhibition (PPI) was first described by Sechenov in 1863 (Sechenov, 1863). Like habituation, it is a pre-attentive mechanism which can reduce the cognitive burden of sensory information. However, unlike habituation, PPI of the startle response is not a learned behaviour (Koch, 1999); it occurs on the first trial. It can improve across days of repeated testing, known as PPI learning (Plappert et al., 2006, Typlt et al., 2013), or be enhanced (or disrupted) through associative conditioning of the prepulse (Li et al., 2009). Another critical distinction between PPI and habituation is that PPI reflects a direct gating of a motor response, in this case the startle response.

PPI occurs when the presentation of a weak pre-stimulus reduces the behavioural response to a strong startling stimulus, the pulse (Peak, 1939; Hoffman and Fleshler, 1963). The strength of this inhibition can vary and is partly dependent on the latency between the pulse and prepulse, termed the interstimulus interval or ISI (Jones and Shannon, 2000a; Bosch and Schmid, 2008). PPI occurs at ISIs ranging between 10-1000 ms. A prepulse can be of the same sensory modality as the startle pulse or PPI can be cross-modal. Current theories suggest that the processing of the prepulse inhibits processing of the pulse, resulting in decreased startle (for schematic representation see to figure 1.2).

The adaptive value of gating neural circuits is proposed to be the prevention of distractive interference during concurrent neural activation, thereby acting as a protective processing mechanism. Essentially, this prevents sensory information from flooding our brain and usurping finite attentional capacities (Braff et al., 1992; Koch and Schnitzler, 1997; Wynn et al., 2004). Another prominent theory regarding the adaptive function of PPI is that the prepulse facilitates

orienting responses such as approach eye-saccades by activating neurons in the Superior Colliculus while simultaneously inhibiting startle-mediating neurons in the brainstem. This is thought to coordinate approach behaviours. By visually orienting to a stimulus and inhibiting eye closure (that accompanies the startle response), PPI can be thought of as a very early form of response selection (Yeomans, 2012).

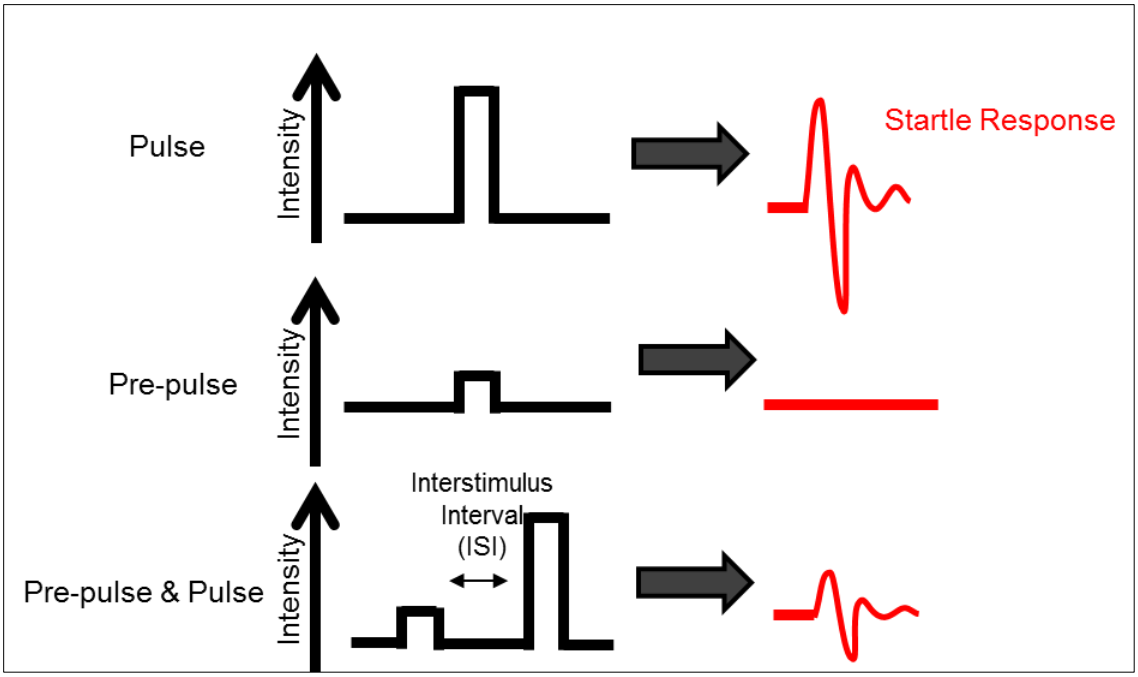

Figure 1.2 Prepulse Inhibition

When a loud, intense acoustic stimulus is presented it induces a robust startle response. If a less intense stimulus is presented, in this case termed the prepulse, it will not induce a startle response. If a prepulse precedes a pulse by 10-1000 ms, this will greatly reduce the magnitude of the startle reflex compared to when the pulse is presented alone. This attenuation of the startle magnitude is referred to as prepulse inhibition (adapted from Koch, 1999)

1.3.1 Prepulse Inhibition vs. Prepulse Facilitation

The presence of a prepulse prior to a startling pulse does not always inhibit the ASR. Prepulse Facilitation (PPF) describes the situation when the presence of a prepulse increases

startle magnitude, compared to when the startling sound is presented alone. Whether PPF or PPI is induced seems to be best predicted by ISI and prepulse type. Very short (>10-15 ms) or very long (<1000 ms) ISIs tend to be the most effective at inducing PPF. While both discrete and continuous prepulses (duration lasting throughout the entire ISI) can induce PPF, continuous prepulses tend to be the most effective –especially at longer ISIs (Graham, 1975; Hsieh et al., 2006).

One feature of both PPF and PPI is that they are disrupted in Schizophrenia (Wynn et al., 2004); however they are thought to be separate, independent processes. They display different time courses, different sensitivity to prepulse saliency, and are modulated differentially by background sound and Gamma-aminobutyric acid (GABA) agonism (Graham, 1975; Ison et al., 1997). They also have opposing effects on the response latency of startle: PPI tends to increase response latency, whereas PPF decreases it (Ison et al., 1973).

The mechanisms and function of PPF have remained elusive. PPF is not due to temporal summation of the prepulse and the startling pulse (Ison et al., 1997). One theory suggests that in PPF the prepulse generally increases arousal, resulting in an increase in startle (Graham, 1975; Reijmers and Peeters, 1994), however there is evidence as well to suggest this may not be the case (Ison et al., 1997). For example, several studies suggest that PPF reflects an orienting or attentional mechanism by which a prepulse stimulus leads to an enhanced startle response (Hazlett et al., 1998; Wynn et al., 2004). Confusingly, PPI has also been suggested to reflect and orienting response (Yeomans, 2012). How two independent and opposing processes may reflect the same orienting response is unclear. One possibility is that they may reflect different aspects, or timing, of the orienting response.

Akin to the dual process theory for habituation, at ISIs close to the border of PPI and PPF, the behavioural output is thought to be balanced between opposing PPF and PPI processes (Reijmers and Peeters, 1994; Ison et al., 1997). Despite the fact that the mechanisms and functions of PPF are largely unknown, because PPF represents a phenomenon that is the behavioural opposite of PPI, it is critical to be aware of the effects this process may have on studies of PPI.

1.3.2 The Neural Circuitry of Prepulse Inhibition

The neuroanatomical circuitry of PPI clearly illustrates its function as a neural gating mechanism. PPI occurs due to the manner in which PPI-mediating structures impinge upon the primary startle pathway. When the pre-pulse is first detected by the ear it excites the Cochlear Root neurons (apart from the ascending hearing pathway, which is not discussed here). It is at this point where the signal is divergently processed through the primary startle pathway as well as by a parallel PPI pathway (see figure 1.3). Within the PPI pathway the signal is transmitted to the inferior and the superior colliculi. Neurons of these nuclei induce activation of the PPT (Kandler and Herbert, 1991). It is supposed that the superior colliculus (SC) integrates and transmits information from all sensory modalities, whereas the inferior colliculus (IC) transmits strictly auditory information to the PPT (Semba and Fibiger, 1992; Fendt et al., 2001). Once activated the PPT is thought to release ACh within the PnC (Koch et al., 1993; Swerdlow and Geyer, 1993). This is the site of imposition that is hypothesized to be critical for PPI of startle. The PPT cholinergic input inhibits startle mediating giant neurons of the PnC (Fendt and Koch, 1999; Bosch and Schmid, 2006), which translates into less activation of the spinal motoneurons resulting in a decreased startle response. This is the major hypothesized mechanism underlying

PPI. New evidence has suggested that there is an additional fast PPI circuit that involves cholinergic projections from the ventral nucleus of the trapezoid body onto cochlear root neurons (Gomez-Nieto et al., 2008; Gomez-Nieto et al., 2014).

It is important to note that the prepulse and pulse are processed in the exact same manner. Due to the ISI, it is thought that the presumed cholinergic inhibition of giant neurons is still in effect when the startle pulse is being processed. Due to this residual inhibition, the PnC is unable to activate the spinal motoneurons as strongly as if the pulse was presented alone. The efficacy of this inhibition is dependent on the duration of the ISI (Jones and Shannon, 2000a; Bosch and Schmid, 2008). Different paths within the PPI circuitry may reflect differential circuits, neurotransmitter systems, and receptors being activated during PPI and playing different roles at different ISIs (Yeomans et al., 2010).

The previously described circuitry is located within the brainstem, however many higher brain structures have also been linked to PPI. These include the hippocampus, amygdala, medial prefrontal cortex, ventral striatum, ventral pallidum, substantia nigra, the ventral tegmental area and the nucleus accumbens, (for review see Koch, 1999). These areas act to modulate PPI via direct, or indirect, projections to the PPT. Figure 1.2.4 illustrates how the PPT is able to act as an integration point for top-down control of PPI. One example for this top-down modulation is the enhancement of PPI in rats and humans by increased attention to the prepulse (Li et al., 2009)

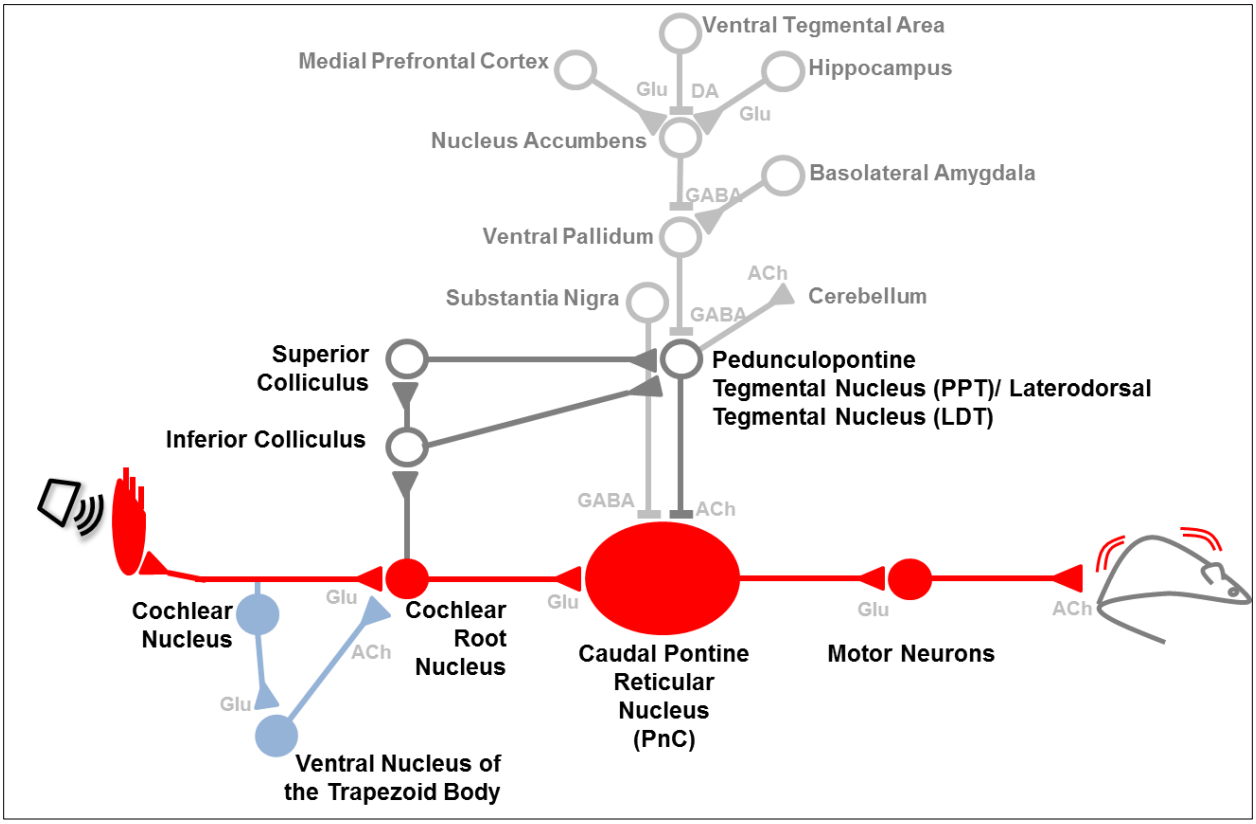

Figure 1.3 The Neural Circuitry of Prepulse Inhibition

This figure displays a simplified neuroanatomical summary of known PPI circuitry. The primary startle circuit is shown in red. The darker grey highlights the structures important for the mediation of PPI, and the lighter grey displays structures that modulate PPI. This diagram illustrates the PPT as the interface for PPI modulation by these higher brain regions. Shown in blue is a fast circuit of PPI suggested by Gomez-Nieto et al. (2014). Both the pulse and prepulse are processed through these pathways in the same manner, but due to the latency between each stimulus, residual (presumed) acetylcholine from the PPT is still inhibiting the PnC during processing of the pulse. This causes a decreased startle response.

1.3.3 Neurotransmitters Involved in Prepulse Inhibition

It was the findings of Koch, Kungel and Herbert (1993) alongside Swerdlow and Geyer (1993), that lead to the principle cholinergic hypothesis of PPI. Both these studies induced general lesions of the PPT, and observed a substantial deficit in PPI. While these seminal studies provided an essential foundation for the field, the methodology was too limited to determine PPT cholinergic input as the singular mediator of PPI. Firstly, the PPT is a heterogeneous structure in terms of neurotransmitter release (Wang and Morales, 2009). Secondly, recent papers have demonstrated that other neurotransmitters play an important role in PPI. Differential modulation of PPI according to the ISI is a prime example of this. Yeomans and colleagues (2010) suggested that ACh transmission mediates PPI at medium to long ISIs (100-1000 ms), but that at shorter ISIs (1-10 ms) GABA is involved. $GABA_A$ antagonism disrupted PPI at short ISIs, whereas $GABA_B$ antagonism disrupted PPI at longer ISIs, suggesting that GABA transmission may be an additional mediator of PPI.

While the field has largely hypothesized that GABA and ACh mediate PPI, other neurotransmitters can modulate PPI at different levels of the neuron circuitry as well. Serotonin modulation has been shown in the hippocampus (Adams and van den Buuse, 2011) and raphe nuclei (Fletcher et al., 2001); whereas dopamine transmission has been heavily implicated in the medial prefrontal cortex (Ellenbroek et al., 1996; Zhang et al., 2000) and striatum (Zhang et al., 2000). Removal of a single neurotransmitter system does not completely ablate PPI. When examining the PPI literature it becomes apparent that even this pre-attentive behaviour is sensitive to changes in the transmission of different neurotransmitters across many different areas of the brain.

1.3.4. Prepulse Inhibition and Acetylcholine

Although PPI is a complex process that is modulated by many neurotransmitter systems, literature within this field has particularly focussed on PPI regulation by ACh. The strongest evidence that ACh is a primary mediator of PPI stems from pharmacology studies. For example, Fendt and Koch (1999) found that cholinergic antagonism within the startle-mediating PnC disrupted PPI, and that general cholinergic agonism enhanced PPI. A choline-free diet also causes drastic reductions in PPI which can be restored by re-introducing a general cholinergic agonist, arecoline, back into the rodent's diet (Wu et al., 1993). Manipulations of the cholinergic system have been well documented to alter PPI, but the cholinergic receptors responsible (discussed below), and the source of cholinergic modulation (discussed in section 1.4.3) remains to be determined.

1.3.4.1 Muscarinic Receptors and Prepulse Inhibition

Systemic muscarinic antagonism using scopolamine disrupted PPI at ISIs of 100 and 300 ms; ISIs of 30 ms or less were unaffected (Jones and Shannon, 2000a). At the cellular level, Bosch and Schmid (2006) found that muscarinic antagonism prevented cholinergic inhibition of the PnC giant neurons *in vitro*. Furthermore, they found that muscarinic M2 and M4 subtype-preferring antagonists mediated this effect. This suggests that the inhibitory, presumably presynaptic M2 and M4 receptors are responsible for the muscarinic component of PPI. However, Bosch and Schmid (2006) did note that the most effective inhibition occurs when general cholinergic agonism is used, suggesting a complementary role of nicotinic receptors.

A lot of emphasis has been placed on understanding how ACh may influence PPI at the level of the PnC. This cholinergic input is assumed to arise from the PPT, and will be discussed

more thoroughly in section 1.4.3. Recently, a novel, 'fast PPI circuit' has been proposed (see figure 1.3), whereby a cholinergic projection from the ventral nucleus of the trapezoid body sends inhibitory cholinergic projections to the cochlear root nucleus (Gomez-Nieto et al., 2014). What receptor types may underlie this inhibition is currently unknown.

There also have been a few studies that have looked at cholinergic modulation of PPI by higher brain structures. Microinfusion of cholinergic agonists into the CA1 and dentate gyrus of the hippocampus disrupted PPI as well as generally reduced startle reactivity. This could be rescued by muscarinic antagonism, suggesting that muscarinic receptors in the hippocampus play a role in modulating PPI (Caine et al., 1992).

1.3.4.2 Nicotinic Receptors and Prepulse Inhibition

Nicotinic acetylcholine receptors (nAChR) are pentameric ligand-gated ion channels. They are can be found within the peripheral and central nervous system. Once activated by endogenous ACh, or an agonist, the channel opens to allow passive flow of positively charged ions through the cell membrane. According to electrochemical gradients this causes an influx of Na^+ and Ca^+, and an efflux of K^+ and overall excitation of the membrane. These channels are composed of a variety of potential subunits. In vertebrates there are 17 known nAChR subunits (α1-10, β1-β4, γ, δ, and ε) and channel composition differs according to the location of the nAChR. General nicotinic agonism, via nicotine, is well known to enhance PPI in human and rodent models (Acri, 1994; Acri et al., 1995; Kumari et al., 1997; Guan et al., 1999).

The most common nAChR expressed in the brain are the α7 and the α4β2 subtypes (Ripoll et al., 2004) and both have been suggested to play a role in PPI. It seems that only α4β2 nAChR

are expressed in PnC giant neurons however, as local, but not systemic α4β2 agonism could improve PPI. In contrast, systemic, but not local, α7 nAChR agonism improved PPI; this suggests that α4β2 nAChRs directly alter startle, whereas there is a modulatory role for α7 nAChR outside the primary startle pathway (Pinnock et al., 2015).

1.3.4.3 A Closer Look at Prepulse Inhibition and the α7 Nicotinic Receptor

This section will take an in depth view of the role the α7 nAChR plays in sensorimotor gating. The α7 nAChR is a homomeric channel, with 5 identical α7 subunits composing the core of the channel (Paterson and Nordberg, 2000). The α7-nAChR rapidly becomes up-regulated and desensitized (Couturier et al., 1990; Fenster et al., 1997) in the persistent presence of ACh (or agonist), and has a lower affinity for ACh than other nAChRs (Hajos and Rogers, 2010). Distinctly, the α7 receptor is more permeable to Ca+ than other nAChRs, particularly α4β2-nAChRs (Seguela et al., 1993). The α7-nAChRs transiently amplifies Ca^{+} signalling by "Ca^{+}-induced Ca^{+}-release" via calcium stores in the endoplasmic reticulum and activation of voltage gated calcium channels (Dajas-Bailador et al., 2002). This signalling pathway is activated by α7-nAChRs at post- and pre-synaptic sites and has been linked to long-term plasticity processes such as modulation of neurotransmitter release, regulation of postsynaptic excitability, and long-term potentiation, (for review see Dajas-Bailador and Wonnacott, 2004). This cellular mechanism clearly illustrates how the α7-nAChR is poised to play an important role in cognition.

There are several different lines of evidence to suggest that that the α7-nAChR is involved in PPI. For example, one of the main behavioural hallmarks of schizophrenia is a deficit in sensorimotor gating. Subsets of schizophrenic patients have abnormalities in the C*HRNA7* gene, which encodes the α7-nAChR. These abnormalities, particularly the dinucleotide polymorphism

in the intron 2 region, have been correlated with deficits in inhibitory gating mechanisms (Freedman et al., 1997; Leonard et al., 2002).

Furthermore, the brains of schizophrenic patients typically have abnormally low levels of α7-nAChRs in the medial prefrontal cortex and hippocampus (Freedman et al., 1995; Guan et al., 1999), both of which are implicated in the neural circuitry of top-down modulation of PPI (see figure 1.3). A DBA/2 mouse model of schizophrenia also correlated a decrease in α7-nAChRs in the hippocampus with the strength of gating deficits (Stevens et al., 1996). Selective agonism of the α7-nAChR restored this mouse model's PPI deficit (Simosky et al., 2001) and can restore many other types of PPI deficits as well (O'Neill et al., 2003; Dunlop et al., 2009; Wallace et al., 2011).

Despite this strong line of pharmacological evidence that the α7-nAChR is an important modulator of PPI, recent studies have shown that α7-nAChR KO mice have normal PPI of the acoustic startle response (Paylor et al., 1998; Young et al., 2011). However, other studies have shown auditory gating deficits in heterozygous $\alpha7^{(-/+)}$ KO mice, as assessed by *in vivo* evoked potential recordings in the hippocampus (Adams et al., 2008). In summary, the current literature suggests that the α7-nAChR is a modulator, rather than a mediator, of PPI, which will be further studied as one aim of this thesis.

1.4 The Pedunculopontine Tegmental Nucleus (PPT)

The PPT, alongside with the parabigeminal (PBG) and LDT nuclei compose the cholinergic cell groups of the midbrain. The LDT and PPT are very similar in structure and connectivity, however, this review will focus on the PPT.

The PPT is a heterogeneous structure in terms of neurotransmitter release. In addition to ACh, GABA (Lavoie and Parent, 1994b), and glutamate (Clements and Grant, 1990) immunoreactivity has been observed in the PPT. There has been debate if these neurotransmitters are co-released or not. This may be in part due to species differences, but in the rodent it appears these neurotransmitters are released independently (Wang and Morales, 2009); however, see (Clements et al., 1991; Lavoie and Parent, 1994b).

A special note regarding the cholinergic neurons of the PPT is that they also release nitric oxide as the all choline acetyltransferase (ChAT) positive neurons co-stain for the nitric oxide marker, β-nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (Vincent et al., 1986; Lavoie and Parent, 1994b). Around 30% of these cholinergic neurons also express substance P markers, corticotropin-releasing factor and gastrin-releasing peptide immunoreactivity (Standaert et al., 1986; Vincent et al., 1986).

1.4.1 Connectivity of the PPT

There are two distinct cholinergic regions within the PPT: the pars dissipatus, which encapsulates the rostral PPT and has sparse ACh neurons; and the pars compactus, which is densely packed with cholinergic neurons and denotes the caudal aspect of the PPT (Martinez-Gonzalez et al., 2011). The anterior vs. posterior PPT receive different input and project to different regions of the brain.

The rostral PPT receives inhibitory GABAergic input from the substania nigra and global pallidus (Granata and Kitai, 1991; Florio et al., 2007). It is also highly innervated by the subthalamic nucleus and ventral tegmental area, and deep cerebellar nuclei (Semba and Fibiger,

1992). The PPT receives the majority of its cortical input from the primary auditory and medial prefrontal cortex (Semba and Fibiger, 1992; Schofield and Motts, 2009). The nucleus basalis, LDT and contralateral PPT seem to innervate the entirety of the PPT. The caudal PPT receives the majority of the incoming sensory input from the ipsilateral SC and IC, as well as ventral cochlear nucleus, principle sensory trigeminal nucleus, and the superior olivary complex (Semba and Fibiger, 1992).

The projections of the cholinergic PPT are diffusely spread through the brain. They can also be dissociated topographically. The anterior PPT innervates the dorsolateral striatum, substania nigra (pars compacta), global pallidus, and nucleus basalis (Semba and Fibiger, 1992; Lavoie and Parent, 1994a; Dautan et al., 2014), whereas the caudal PPT preferentially innervates the dorsal striatum and nucleus accumbens, IC, SC, ventral tegmental area, nucleus pontis oralis, reticular pontine formation, PnC, and thalamus (Semba et al., 1990; Koch et al., 1993; Lavoie and Parent, 1994a; Garcia-Rill et al., 2001; Dautan et al., 2014). Interestingly, the same cholinergic neurons innervate both the PnC and thalamus (Semba et al., 1990).

1.4.2 Function of the PPT

The PPT has been implicated in a number of different behavioural and cognitive function. As rostral vs. caudal regions display a distinct pattern of afferent and efferent connectivity, it is no surprise that there is a topographical hypothesis in the functioning of the PPT (see Martinez-Gonzalez et al., 2011; Gut and Winn, 2016 for more detail). However, this review will focus on the general hypothesized function of the PPT as a whole.

Cholinergic projections from the PPT to the thalamus encompass an arm of the ascending reticular activating system which mediates wakefulness and sleep transitions (Walter, 2014). Cholinergic PPT neurons are activated during rapid eye movement (REM) sleep (Hobson et al., 1975) and optogenetic activation of these neurons is sufficient to induce REM sleep (Van Dort et al., 2015). It is thought these cholinergic neurons drive state-transitions by producing transient responses to sensory events (Petzold et al., 2015). While induction of REM sleep might be mediated by projections to the nucleus pontis oralis (Nguyen et al., 2013), the cholinergic PPT is well poised to influence arousal and cortical processing through its connections.

It is suggested that the PPT has a greater influence on cognitive control than previously thought. Historically the PPT was highly implicated in motor control, specifically locomotion. However, after extensive review of animal studies following PPT lesions (that generally show no gross motor impairments), Gut and Winn (2016) suggest that the PPT can be more appropriately thought as a part of a lower-level action selection process. Due to its rapid detection of sensory information, and output to striatal and thalamic inputs, Gut and Winn predict the PPT prevents impulsive and inappropriate responding. Accordingly, a recent study found that while rats with selective cholinergic lesions of the PPT showed little impairment in a 5-choice serial reaction time task, the only notable difference in their behaviour was a tendency towards more impulsive responding (Cyr et al., 2015).

Expanding this idea further, the PPT may also play a role in associating the outcome of actions with outcomes or environment. The PPT is well known to be implicated in reward-based learning as lesions to this area disrupt morphine-induced (Olmstead and Franklin, 1993) and nicotine-induced conditioned place preference (Laviolette et al., 2002). Modulation of PPT

activity can differentially influence the activity of the ventral tegmental area (Pan and Hyland, 2005; Xiao et al., 2016), particularly when associated with a sensory cue (Pan and Hyland, 2005). This may suggest that the PPT can execute basic action selection and associate this with reward. Fittingly, recent studies have shown that neuronal activity in mouse PPT reflects both action and outcome in a decision-making task (Thompson and Felsen, 2013) indicating that the PPT's role in action-selection could adapt with experience.

1.4.3 The Cholinergic Midbrain and its Role in Sensorimotor Gating

The idea that the PPT may mediate early action selection fits excellently with its hypothesized role in PPI. As stated, PPI has also been suggested to be an early form of action selection, mainly though promoting orienting responses (eye saccades) while inhibiting startle and eye-closure (Yeomans, 2012). The execution of these functions is predicted to be mediated via the PPT. It sends cholinergic projections to the SC which can influence eye saccades (Kobayashi et al., 2002), and as discussed, it is known to innervate and inhibit the startle mediating brainstem (Koch et al., 1993; Bosch and Schmid, 2008).

The longstanding hypothesis that the mechanism underlying PPI stems from cholinergic inhibition of startle-mediating neurons via the PPT has been supported by a large body of evidence. As previously discussed this primarily comes from a seminal study by Koch, Kungel and Herbert (1993), where they lesioned the PPT and saw a disruption in PPI but no difference in baseline startle magnitude. They inferred this was mediated by diminished cholinergic input from the PPT, as they observed 35% decrease in ChAT immunoreactivity in the PPT. Similar findings were reported by Swerdlow and Geyer (1993) using electrolytic lesions, however they also

reported an increase in startle magnitude (when no prepulse was present). Fittingly, microinfusions of cholinergic agonists into the PnC enhanced PPI, and muscarinic antagonism disrupted it (Fendt and Koch, 1999). The idea that this cholinergic modulation stemmed from the PPT was further supported by *in vitro* stimulation of PPT projections and sensory afferents to the PnC which caused a delayed inhibition of synaptic transmission (Bosch and Schmid, 2008). Together, the evidence strongly supports that the cholinergic cells of PPT mediate PPI.

However, a recent study by MacLaren and colleagues (2014) had the unique advantage to selectively lesion the cholinergic cells of the PPT using a urotensin II diptheria toxin fusion protein. Following this, they found no disruption in PPI, but profoundly reduced baseline startle magnitudes. When they completed a general lesion of the PPT, they could re-affirm past studies showing disruptions in PPI. Additionally, a mouse with a conditional knock-out of cholinergic transmission in the midbrain (LDT, PPT and PGB) displayed improved PPI compared to wildtype mice, with no change in baseline startle magnitude (Machold, 2013). These studies represent some of the first evidence to suggest that it is not the cholinergic cells of the PPT that are critical for PPI. However, it is impossible to rule out compensatory mechanisms that accompany lesion techniques or knock-out models (for review see Barbaric et al., 2007; Otchy et al., 2015).

Although *in vitro* patch clamp studies in the rodent have shown that cholinergic agonism can inhibit synaptic signals in startle-mediating giant neurons of the PnC fitting with cholinergic midbrain mediation of PPI (Bosch and Schmid, 2006, 2008); *in vitro* recordings of unidentified PnC neurons in the cat following electrical stimulation of the PPT caused excitatory prolonged responses (a train of action potentials lasting greater than 12 ms following stimulation). This could be blocked by administration of scopolamine, a muscarinic antagonist, and induced using

carbachol, a general ACh agonist (Homma et al., 2002). Interestingly, this prolonged responsiveness seems similar to that observed during *in vivo* recordings of PnC neurons following application of Substance P. Furthermore, as previously discussed, activity in PnC neurons in response to to acoustic stimulation was also enhanced by cholinergic agonism in rats (Kungel et al., 1994). This suggests that ACh may have an excitatory influence on startle mediating neurons. How this influences the output of the PnC (and ultimately behaviour) remains unanswered. In light of this recent evidence, it is clear that the midbrain cholinergic hypothesis of PPI may need to be re-evaluated, specifically using techniques that can manipulate neuronal activity with improved cell-type and temporal specificity.

1.5 Rationale and Hypothesis

In this thesis, I aimed to understand the differential role ACh may play in sensorimotor gating and the sensory filtering of reflexive vs. non-reflexive behaviours. Based on the literature review above, my overall hypothesis is that ACh is critical for sensorimotor gating of the ASR, and habituation of locomotor behaviour. It seems to play no role in the short-term habituation of the ASR, however, ACh's role within long-term habituation of the ASR has been largely unexplored. Understanding the role of ACh in sensory filtering and sensorimotor gating is important for several reasons. Firstly, these processes are disrupted in a number of psychiatric illnesses and better treatment options may result from understanding the underlying pharmacological mechanisms related to these processes. Secondly, sensory filtering and sensorimotor gating are thought to act as proxy measures for an individual's efficiency of information processing and/or methods for reducing cognitive burden. Uncovering the subtle differences in modulation of these

processes will help us not only to better understand the processes themselves, but how they relate to higher-cognitive function.

My aim was to re-define the role of cholinergic modulation of sensory filtering and sensorimotor gating. Next, we aimed to refine this by investigating what cholinergic receptor is critical for modulation of these processes, as well as what nucleus was providing the necessary cholinergic input.

Chapter 2 defines the role of ACh on PPI and habituation of the ASR using transgenic mice with a reduced cholinergic tone. **Hypothesis:** I hypothesize that ACh is the primary mediator of prepulse inhibition, but plays no role in habituation of the acoustic startle reflex. Mice with deficient cholinergic tone mice will show a disruption on PPI, but intact habituation.

Chapter 3 uncovers the receptors mediating cholinergic modulation of sensorimotor gating mechanisms. Specifically, I investigated the α7-nAChR's role, using an α7-nAChR knock-out mouse model. **Hypothesis A:** I hypothesized that the α7-nAChR is involved in PPI of startle, and predict these mice will have a deficit in PPI. Additionally, I hypothesized that this receptor is necessary for nicotine-induced enhancement of PPI, but not involved in the short-term or long-term habituation of startle. **Hypothesis B:** If deficits in pre-attentive cognitive mechanisms like sensorimotor gating were observed, I predicted these deficits would lead to disruptions in higher cognitive function, such as working memory in spatial tasks (e.g. the Barnes Maze).

In Chapter 4 I aimed to evaluate the role of cholinergic PPT neurons in sensorimotor gating using designer receptors exclusively activated by designer drugs (DREADDs) and optogenetics. Using these complementary techniques I both transiently inhibited (DREADDs) and

activated (optogenetics) cholinergic cells specifically in the PPT to elucidate the role of this nucleus. **Hypothesis**: Based on findings from Chapter 2, I hypothesized that pulsatile cholinergic release from the PPT is responsible for the cholinergic modulation of PPI. I predicted that by using DREADDs to selectively inhibit these cells, I would observe a reduction in PPI. Using the opposite approach, I predicted that optogenetic stimulation of cholinergic PPT neurons would induce PPI (in place of an auditory prepulse) prior to a startling stimulus.

1.6 References

Acri JB (1994) Nicotine modulates effects of stress on acoustic startle reflexes in rats: dependence on dose, stressor and initial reactivity. Psychopharmacology (Berl) 116:255-265.

Acri JB, Brown KJ, Saah MI, Grunberg NE (1995) Strain and age differences in acoustic startle responses and effects of nicotine in rats. Pharmacol Biochem Behav 50:191-198.

Adams CE, Yonchek JC, Zheng L, Collins AC, Stevens KE (2008) Altered hippocampal circuit function in C3H alpha7 null mutant heterozygous mice. Brain Res 1194:138-145.

Adams W, van den Buuse M (2011) Hippocampal serotonin depletion facilitates the enhancement of prepulse inhibition by risperidone: Possible role of 5-HT2C receptors in the dorsal hippocampus. Neuropharmacology 61:458-467.

Barbaric I, Miller G, Dear TN (2007) Appearances can be deceiving: phenotypes of knockout mice. Brief Funct Genomic Proteomic 6:91-103.

Barmack NH, Baughman RW, Eckenstein FP (1992a) Cholinergic innervation of the cerebellum of rat, rabbit, cat, and monkey as revealed by choline acetyltransferase activity and immunohistochemistry. J Comp Neurol 317:233-249.

Barmack NH, Baughman RW, Eckenstein FP, Shojaku H (1992b) Secondary vestibular cholinergic projection to the cerebellum of rabbit and rat as revealed by choline acetyltransferase immunohistochemistry, retrograde and orthograde tracers. J Comp Neurol 317:250-270.

Best AR, Thompson JV, Fletcher ML, Wilson DA (2005) Cortical metabotropic glutamate receptors contribute to habituation of a simple odor-evoked behavior. J Neurosci 25:2513-2517.

Bosch D, Schmid S (2006) Activation of muscarinic cholinergic receptors inhibits giant neurones in the caudal pontine reticular nucleus. Eur J Neurosci 24:1967-1975.

Bosch D, Schmid S (2008) Cholinergic mechanism underlying prepulse inhibition of the startle response in rats. Neuroscience 155:326-335.

Braff DL, Geyer MA (1990) Sensorimotor gating and schizophrenia. Human and animal model studies. Arch Gen Psychiatry 47:181-188.

Braff DL, Grillon C, Geyer MA (1992) Gating and habituation of the startle reflex in schizophrenic patients. Arch Gen Psychiatry 49:206-215.

Braff DL, Geyer MA, Swerdlow NR (2001) Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology (Berl) 156:234-258.

Brodkin J (1999) Assessing memory in mice using habituation of nose-poke responding. Behav Pharmacol 10:445-451.

Brown CP (1976) Two types of habituation in chicks: differential dependence on cholinergic activity. Pharmacol Biochem Behav 4:235-238.

Caine SB, Geyer MA, Swerdlow NR (1992) Hippocampal modulation of acoustic startle and prepulse inhibition in the rat. Pharmacol Biochem Behav 43:1201-1208.

Clements JR, Grant S (1990) Glutamate-like immunoreactivity in neurons of the laterodorsal tegmental and pedunculopontine nuclei in the rat. Neurosci Lett 120:70-73.

Clements JR, Toth DD, Highfield DA, Grant SJ (1991) Glutamate-like immunoreactivity is present within cholinergic neurons of the laterodorsal tegmental and pedunculopontine nuclei. Adv Exp Med Biol 295:127-142.

Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar n, Valera S, Barkas T, Ballivet M (1990) A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron 5:847-856.

Cyr M, Parent MJ, Mechawar n, Rosa-Neto P, Soucy JP, Clark SD, Aghourian M, Bedard MA (2015) Deficit in sustained attention following selective cholinergic lesion of the pedunculopontine tegmental nucleus in rat, as measured with both post-mortem immunocytochemistry and in vivo PET imaging with [(1)(8)F]fluoroethoxybenzovesamicol. Behav Brain Res 278:107-114.

Dajas-Bailador F, Wonnacott S (2004) Nicotinic acetylcholine receptors and the regulation of neuronal signalling. Trends Pharmacol Sci 25:317-324.

Dajas-Bailador FA, Mogg AJ, Wonnacott S (2002) Intracellular Ca2+ signals evoked by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca2+ channels and Ca2+ stores. J Neurochem 81:606-614.

Dautan D, Huerta-Ocampo I, Witten IB, Deisseroth K, Bolam JP, Gerdjikov T, Mena-Segovia J (2014) A major external source of cholinergic innervation of the striatum and nucleus accumbens originates in the brainstem. J Neurosci 34:4509-4518.

Davis M (1970) Effects of interstimulus interval length and variability on startle-response habituation in the rat. J Comp Physiol Psychol 72:177-192.

Davis M (1984) The mammalian startle response. New York, London: Plenum Press.

Davis M, Gendelman DS, Tischler MD, Gendelman PM (1982a) A primary acoustic startle circuit: lesion and stimulation studies. J Neurosci 2:791-805.

Davis M, Parisi T, Gendelman DS, Tischler M, Kehne JH (1982b) Habituation and sensitization of startle reflexes elicited electrically from the brainstem. Science 218:688-690.

Dunlop J, Lock T, Jow B, Sitzia F, Grauer S, Jow F, Kramer A, Bowlby MR, Randall A, Kowal D, Gilbert A, Comery TA, Larocque J, Soloveva V, Brown J, Roncarati R (2009) Old and new pharmacology: positive allosteric modulation of the alpha7 nicotinic acetylcholine receptor by the 5-hydroxytryptamine(2B/C) receptor antagonist SB-206553 (3,5-dihydro-5-methyl-n-3-pyridinylbenzo[1,2-b:4,5-b']di pyrrole-1(2H)-carboxamide). J Pharmacol Exp Ther 328:766-776.

Ellenbroek BA, Budde S, Cools AR (1996) Prepulse inhibition and latent inhibition: the role of dopamine in the medial prefrontal cortex. Neuroscience 75:535-542.

Fendt M, Koch M (1999) Cholinergic modulation of the acoustic startle response in the caudal pontine reticular nucleus of the rat. Eur J Pharmacol 370:101-107.

Fendt M, Koch M, Schnitzler HU (1994) Lesions of the central gray block the sensitization of the acoustic startle response in rats. Brain Res 661:163-173.

Fendt M, Li L, Yeomans JS (2001) Brain stem circuits mediating prepulse inhibition of the startle reflex. Psychopharmacology (Berl) 156:216-224.

Fenster CP, Rains MF, Noerager B, Quick MW, Lester RA (1997) Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine. J Neurosci 17:5747-5759.
Fletcher PJ, Selhi ZF, Azampanah A, Sills TL (2001) Reduced brain serotonin activity disrupts prepulse inhibition of the acoustic startle reflex. Effects of 5,7-dihydroxytryptamine and p-chlorophenylalanine. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 24:399-409.
Florio T, Scarnati E, Confalone G, Minchella D, Galati S, Stanzione P, Stefani A, Mazzone P (2007) High-frequency stimulation of the subthalamic nucleus modulates the activity of pedunculopontine neurons through direct activation of excitatory fibres as well as through indirect activation of inhibitory pallidal fibres in the rat. Eur J Neurosci 25:1174-1186.
Frankland PW, Yeomans JS (1995) Fear-potentiated startle and electrically evoked startle mediated by synapses in rostrolateral midbrain. Behav Neurosci 109:669-680.
Frankland PW, Scott BW, Yeomans JS (1995) Axons and synapses mediating electrically evoked startle: collision tests and latency analysis. Brain Res 670:97-111.
Freedman R, Hall M, Adler LE, Leonard S (1995) Evidence in postmortem brain tissue for decreased numbers of hippocampal nicotinic receptors in schizophrenia. Biol Psychiatry 38:22-33.
Freedman R, Adler LE, Bickford P, Byerley W, Coon H, Cullum CM, Griffith JM, Harris JG, Leonard S, Miller C, et al. (1994) Schizophrenia and nicotinic receptors. Harv Rev Psychiatry 2:179-192.
Freedman R et al. (1997) Linkage of a neurophysiological deficit in schizophrenia to a chromosome 15 locus. Proc Natl Acad Sci U S A 94:587-592.
Freudenberg F, Dieckmann M, Winter S, Koch M, Schwabe K (2007) Selective breeding for deficient sensorimotor gating is accompanied by increased perseveration in rats. Neuroscience 148:612-622.
Garcia-Rill E, Skinner RD, Miyazato H, Homma Y (2001) Pedunculopontine stimulation induces prolonged activation of pontine reticular neurons. Neuroscience 104:455-465.
Geyer MA (2006) The family of sensorimotor gating disorders: comorbidities or diagnostic overlaps? Neurotox Res 10:211-220.
Giovannini MG, Rakovska A, Benton RS, Pazzagli M, Bianchi L, Pepeu G (2001) Effects of novelty and habituation on acetylcholine, GABA, and glutamate release from the frontal cortex and hippocampus of freely moving rats. Neuroscience 106:43-53.
Gogan P (1970) The startle and orienting reactions in man. A study of their characteristics and habituation. Brain Res 18:117-135.
Gomez-Nieto R, Rubio ME, Lopez DE (2008) Cholinergic input from the ventral nucleus of the trapezoid body to cochlear root neurons in rats. J Comp Neurol 506:452-468.
Gomez-Nieto R, Sinex DG, Horta-Junior Jde A, Castellano O, Herrero-Turrion JM, Lopez DE (2014) A fast cholinergic modulation of the primary acoustic startle circuit in rats. Brain Struct Funct 219:1555-1573.
Graham FK (1975) Presidential Address, 1974. The more or less startling effects of weak prestimulation. Psychophysiology 12:238-248.

Granata AR, Kitai ST (1991) Inhibitory substantia nigra inputs to the pedunculopontine neurons. Exp Brain Res 86:459-466.
Groves PM, Wilson CJ, Boyle RD (1974) Brain stem pathways, cortical modulation, and habituation of the acoustic startle response. Behav Biol 10:391-418.
Groves PM, Glanzman DL, Patterson MM, Thompson RF (1970) Excitability of cutaneous afferent terminals during habituation and sensitization in acute spinal cat. Brain Res 18:388-392.
Guan ZZ, Zhang X, Blennow K, Nordberg A (1999) Decreased protein level of nicotinic receptor alpha7 subunit in the frontal cortex from schizophrenic brain. Neuroreport 10:1779-1782.
Gut NK, Winn P (2016) The pedunculopontine tegmental nucleus-A functional hypothesis from the comparative literature. Mov Disord 31:615-624.
Hajos M, Rogers BN (2010) Targeting alpha7 nicotinic acetylcholine receptors in the treatment of schizophrenia. Curr Pharm Des 16:538-554.
Hazlett EA, Romero MJ, Haznedar MM, New AS, Goldstein KE, Newmark RE, Siever LJ, Buchsbaum MS (2007) Deficient attentional modulation of startle eyeblink is associated with symptom severity in the schizophrenia spectrum. Schizophr Res 93:288-295.
Hazlett EA, Buchsbaum MS, Haznedar MM, Singer MB, Germans MK, Schnur DB, Jimenez EA, Buchsbaum BR, Troyer BT (1998) Prefrontal cortex glucose metabolism and startle eyeblink modification abnormalities in unmedicated schizophrenia patients. Psychophysiology 35:186-198.
Hitchcock J, Davis M (1986) Lesions of the amygdala, but not of the cerebellum or red nucleus, block conditioned fear as measured with the potentiated startle paradigm. Behav Neurosci 100:11-22.
Hoenig K, Hochrein A, Quednow BB, Maier W, Wagner M (2005) Impaired prepulse inhibition of acoustic startle in obsessive-compulsive disorder. Biol Psychiatry 57:1153-1158.
Hoffman HS, Fleshler M (1963) Startle Reaction: Modification by Background Acoustic Stimulation. Science 141:928-930.
Homma Y, Skinner RD, Garcia-Rill E (2002) Effects of pedunculopontine nucleus (PPN) stimulation on caudal pontine reticular formation (PnC) neurons in vitro. J Neurophysiol 87:3033-3047.
Hsieh MH, Swerdlow NR, Braff DL (2006) Effects of background and prepulse characteristics on prepulse inhibition and facilitation: implications for neuropsychiatric research. Biol Psychiatry 59:555-559.
Hughes RN (1984) Cholinergic and monoaminergic substrates of startle habituation. Life Sci 34:2101-2105.
Ikegami S (1994) Behavioral impairment in radial-arm maze learning and acetylcholine content of the hippocampus and cerebral cortex in aged mice. Behavioural brain research 65:103-111.
Ison JR, McAdam DW, Hammond GR (1973) Latency and amplitude changes in the acoustic startle reflex of the rat produced by variation in auditory prestimulation. Physiol Behav 10:1035-1039.

Ison JR, Taylor MK, Bowen GP, Schwarzkopf SB (1997) Facilitation and inhibition of the acoustic startle reflex in the rat after a momentary increase in background noise level. Behav Neurosci 111:1335-1352.
Jones CK, Shannon HE (2000) Muscarinic cholinergic modulation of prepulse inhibition of the acoustic startle reflex. J Pharmacol Exp Ther 294:1017-1023.
Kobayashi Y, Inoue Y, Yamamoto M, Isa T, Aizawa H (2002) Contribution of pedunculopontine tegmental nucleus neurons to performance of visually guided saccade tasks in monkeys. J Neurophysiol 88:715-731.
Koch M (1999) The neurobiology of startle. Prog Neurobiol 59:107-128.
Koch M, Schnitzler HU (1997) The acoustic startle response in rats--circuits mediating evocation, inhibition and potentiation. Behav Brain Res 89:35-49.
Koch M, Lingenhohl K, Pilz PK (1992) Loss of the acoustic startle response following neurotoxic lesions of the caudal pontine reticular formation: possible role of giant neurons. Neuroscience 49:617-625.
Koch M, Kungel M, Herbert H (1993) Cholinergic neurons in the pedunculopontine tegmental nucleus are involved in the mediation of prepulse inhibition of the acoustic startle response in the rat. Exp Brain Res 97:71-82.
Krase W, Koch M, Schnitzler HU (1994) Substance P is involved in the sensitization of the acoustic startle response by footshocks in rats. Behav Brain Res 63:81-88.
Kumari V, Cotter PA, Checkley SA, Gray JA (1997) Effect of acute subcutaneous nicotine on prepulse inhibition of the acoustic startle reflex in healthy male non-smokers. Psychopharmacology (Berl) 132:389-395.
Kungel M, Koch M, Friauf E (1996) Cysteamine impairs the development of the acoustic startle response in rats: possible role of somatostatin. Neurosci Lett 202:181-184.
Kungel M, Ebert U, Herbert H, Ostwald J (1994) Substance P and other putative transmitters modulate the activity of reticular pontine neurons: an electrophysiological and immunohistochemical study. Brain Res 643:29-39.
Lamprea MR, Cardenas FP, Silveira R, Walsh TJ, Morato S (2003) Effects of septal cholinergic lesion on rat exploratory behavior in an open-field. Braz J Med Biol Res 36:233-238.
Laviolette SR, Alexson TO, van der Kooy D (2002) Lesions of the tegmental pedunculopontine nucleus block the rewarding effects and reveal the aversive effects of nicotine in the ventral tegmental area. J Neurosci 22:8653-8660.
Lavoie B, Parent A (1994a) Pedunculopontine nucleus in the squirrel monkey: projections to the basal ganglia as revealed by anterograde tract-tracing methods. J Comp Neurol 344:210-231.
Lavoie B, Parent A (1994b) Pedunculopontine nucleus in the squirrel monkey: distribution of cholinergic and monoaminergic neurons in the mesopontine tegmentum with evidence for the presence of glutamate in cholinergic neurons. J Comp Neurol 344:190-209.
Leaton RN, Supple WF, Jr. (1986) Cerebellar vermis: essential for long-term habituation of the acoustic startle response. Science 232:513-515.
Leaton RN, Cassella JV, Borszcz GS (1985) Short-term and long-term habituation of the acoustic startle response in chronic decerebrate rats. Behav Neurosci 99:901-912.

Leonard S, Gault J, Hopkins J, Logel J, Vianzon R, Short M, Drebing C, Berger R, Venn D, Sirota P, Zerbe G, Olincy A, Ross RG, Adler LE, Freedman R (2002) Association of promoter variants in the alpha7 nicotinic acetylcholine receptor subunit gene with an inhibitory deficit found in schizophrenia. Arch Gen Psychiatry 59:1085-1096.

Li L, Du Y, Li n, Wu X, Wu Y (2009) Top-down modulation of prepulse inhibition of the startle reflex in humans and rats. Neurosci Biobehav Rev 33:1157-1167.

Lingenhohl K, Friauf E (1992) Giant neurons in the caudal pontine reticular formation receive short latency acoustic input: an intracellular recording and HRP-study in the rat. J Comp Neurol 325:473-492.

Lingenhohl K, Friauf E (1994) Giant neurons in the rat reticular formation: a sensorimotor interface in the elementary acoustic startle circuit? J Neurosci 14:1176-1194.

Ludewig K, Geyer MA, Vollenweider FX (2003) Deficits in prepulse inhibition and habituation in never-medicated, first-episode schizophrenia. Biol Psychiatry 54:121-128.

Ludewig K, Geyer MA, Etzensberger M, Vollenweider FX (2002) Stability of the acoustic startle reflex, prepulse inhibition, and habituation in schizophrenia. Schizophr Res 55:129-137.

Machold RP (2013) Loss of rostral brainstem cholinergic activity results in decreased ultrasonic vocalization behavior and altered sensorimotor gating. Behav Brain Res 256:51-55.

MacLaren DA, Markovic T, Clark SD (2014) Assessment of sensorimotor gating following selective lesions of cholinergic pedunculopontine neurons. The European journal of neuroscience 40:3526-3537.

Markou A, Matthews K, Overstreet DH, Koob GF, Geyer MA (1994) Flinders resistant hypocholinergic rats exhibit startle sensitization and reduced startle thresholds. Biol Psychiatry 36:680-688.

Martinez-Gonzalez C, Bolam JP, Mena-Segovia J (2011) Topographical organization of the pedunculopontine nucleus. Front Neuroanat 5:22.

Muller U, Cristina n, Li ZW, Wolfer DP, Lipp HP, Rulicke T, Brandner S, Aguzzi A, Weissmann C (1994) Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene. Cell 79:755-765.

Nguyen TQ, Liang CL, Marks GA (2013) GABA(A) receptors implicated in REM sleep control express a benzodiazepine binding site. Brain Res 1527:131-140.

Nielsen DM, Derber WJ, McClellan DA, Crnic LS (2002) Alterations in the auditory startle response in Fmr1 targeted mutant mouse models of fragile X syndrome. Brain Res 927:8-17.

O'Neill HC, Rieger K, Kem WR, Stevens KE (2003) DMXB, an alpha7 nicotinic agonist, normalizes auditory gating in isolation-reared rats. Psychopharmacology (Berl) 169:332-339.

Oliveras I, Rio-Alamos C, Canete T, Blazquez G, Martinez-Membrives E, Giorgi O, Corda MG, Tobena A, Fernandez-Teruel A (2015) Prepulse inhibition predicts spatial working memory performance in the inbred Roman high- and low-avoidance rats and in genetically heterogeneous NIH-HS rats: relevance for studying pre-attentive and cognitive anomalies in schizophrenia. Front Behav Neurosci 9:213.

Olmstead MC, Franklin KB (1993) Effects of pedunculopontine tegmental nucleus lesions on morphine-induced conditioned place preference and analgesia in the formalin test. Neuroscience 57:411-418.

Ornitz EM, Lane SJ, Sugiyama T, de Traversay J (1993) Startle modulation studies in autism. J Autism Dev Disord 23:619-637.
Otchy TM, Wolff SB, Rhee JY, Pehlevan C, Kawai R, Kempf A, Gobes SM, Olveczky BP (2015) Acute off-target effects of neural circuit manipulations. Nature 528:358-363.
Pan WX, Hyland BI (2005) Pedunculopontine tegmental nucleus controls conditioned responses of midbrain dopamine neurons in behaving rats. Journal of Neuroscience 25:4725-4732.
Parwani A, Duncan EJ, Bartlett E, Madonick SH, Efferen TR, Rajan R, Sanfilipo M, Chappell PB, Chakravorty S, Gonzenbach S, Ko GN, Rotrosen JP (2000) Impaired prepulse inhibition of acoustic startle in schizophrenia. Biol Psychiatry 47:662-669.
Paterson D, Nordberg A (2000) Neuronal nicotinic receptors in the human brain. Prog Neurobiol 61:75-111.
Paylor R, Nguyen M, Crawley JN, Patrick J, Beaudet A, Orr-Urtreger A (1998) Alpha7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensorimotor gating: a behavioral characterization of Acra7-deficient mice. Learn Mem 5:302-316.
Peak H (1939) Time order error in successive judgments and in reflexes. I. Inhibition of the judgment and the reflex. Journal of Experimental Psychology 25:535-565.
Perry W, Minassian A, Lopez B, Maron L, Lincoln A (2007) Sensorimotor gating deficits in adults with autism. Biol Psychiatry 61:482-486.
Petzold A, Valencia M, Pal B, Mena-Segovia J (2015) Decoding brain state transitions in the pedunculopontine nucleus: cooperative phasic and tonic mechanisms. Front Neural Circuits 9:68.
Pilz PK, Schnitzler HU (1996) Habituation and sensitization of the acoustic startle response in rats: amplitude, threshold, and latency measures. Neurobiol Learn Mem 66:67-79.
Pilz PK, Arnold SW, Rischawy AT, Plappert CF (2014) Longterm-habituation of the startle response in mice is stimulus modality, but not context specific. Frontiers in integrative neuroscience 7:103.
Pinnock F, Bosch D, Brown T, Simons n, Yeomans JR, DeOliveira C, Schmid S (2015) Nicotine receptors mediating sensorimotor gating and its enhancement by systemic nicotine. Frontiers in behavioral neuroscience 9:30.
Plappert CF, Kuhn S, Schnitzler HU, Pilz PK (2006) Experience increases the prepulse inhibition of the acoustic startle response in mice. Behav Neurosci 120:16-23.
Poon CS, Schmid S (2012) Non-associative learning In: Encyclopedia of the Sciences of Learning (Seel NM, ed), pp 2475-2477. Heidelberg: Springer.
Rankin CH (2009) Introduction to special issue of neurobiology of learning and memory on habituation. Neurobiol Learn Mem 92:125-126.
Rankin CH, Abrams T, Barry RJ, Bhatnagar S, Clayton DF, Colombo J, Coppola G, Geyer MA, Glanzman DL, Marsland S, McSweeney FK, Wilson DA, Wu CF, Thompson RF (2009) Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. Neurobiol Learn Mem 92:135-138.
Reijmers LG, Peeters BW (1994) Effects of acoustic prepulses on the startle reflex in rats: a parametric analysis. Brain Res 667:144-150.

Ripoll n, Bronnec M, Bourin M (2004) Nicotinic receptors and schizophrenia. Curr Med Res Opin 20:1057-1074.
Rosen JB, Davis M (1988) Enhancement of acoustic startle by electrical stimulation of the amygdala. Behav Neurosci 102:195-202, 324.
Sanderson DJ, Bannerman DM (2011) Competitive short-term and long-term memory processes in spatial habituation. J Exp Psychol Anim Behav Process 37:189-199.
Schildein S, Huston JP, Schwarting RK (2002) Open field habituation learning is improved by nicotine and attenuated by mecamylamine administered posttrial into the nucleus accumbens. Neurobiol Learn Mem 77:277-290.
Schofield BR, Motts SD (2009) Projections from auditory cortex to cholinergic cells in the midbrain tegmentum of guinea pigs. Brain Res Bull 80:163-170.
Sechenov IM (1863) Reflexes of the Brain. Massachusetts: M.I.T. Press.
Seguela P, Wadiche J, Dineley-Miller K, Dani JA, Patrick JW (1993) Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. J Neurosci 13:596-604.
Semba K, Fibiger HC (1992) Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: a retro- and antero-grade transport and immunohistochemical study. J Comp Neurol 323:387-410.
Semba K, Reiner PB, Fibiger HC (1990) Single cholinergic mesopontine tegmental neurons project to both the pontine reticular formation and the thalamus in the rat. Neuroscience 38:643-654.
Sheets LP, Dean KF, Reiter LW (1988) Ontogeny of the acoustic startle response and sensitization to background noise in the rat. Behav Neurosci 102:706-713.
Simons-Weidenmaier NS, Weber M, Plappert CF, Pilz PK, Schmid S (2006) Synaptic depression and short-term habituation are located in the sensory part of the mammalian startle pathway. BMC Neurosci 7:38.
Simosky JK, Stevens KE, Kem WR, Freedman R (2001) Intragastric DMXB-A, an alpha7 nicotinic agonist, improves deficient sensory inhibition in DBA/2 mice. Biological psychiatry 50:493-500.
Singer P, Hauser J, Llano Lopez L, Peleg-Raibstein D, Feldon J, Gargiulo PA, Yee BK (2013) Prepulse inhibition predicts working memory performance whilst startle habituation predicts spatial reference memory retention in C57BL/6 mice. Behav Brain Res 242:166-177.
Standaert DG, Saper CB, Rye DB, Wainer BH (1986) Colocalization of atriopeptin-like immunoreactivity with choline acetyltransferase- and substance P-like immunoreactivity in the pedunculopontine and laterodorsal tegmental nuclei in the rat. Brain Res 382:163-168.
Stevens KE, Freedman R, Collins AC, Hall M, Leonard S, Marks MJ, Rose GM (1996) Genetic correlation of inhibitory gating of hippocampal auditory evoked response and alpha-bungarotoxin-binding nicotinic cholinergic receptors in inbred mouse strains. Neuropsychopharmacology 15:152-162.
Swerdlow NR, Geyer MA (1993) Prepulse inhibition of acoustic startle in rats after lesions of the pedunculopontine tegmental nucleus. Behav Neurosci 107:104-117.

Swerdlow NR, Light GA, Cadenhead KS, Sprock J, Hsieh MH, Braff DL (2006) Startle gating deficits in a large cohort of patients with schizophrenia: relationship to medications, symptoms, neurocognition, and level of function. Arch Gen Psychiatry 63:1325-1335.
Takahashi H et al. (2008) Impaired prepulse inhibition and habituation of acoustic startle response in Japanese patients with schizophrenia. Neurosci Res 62:187-194.
Thiel CM, Huston JP, Schwarting RK (1998) Hippocampal acetylcholine and habituation learning. Neuroscience 85:1253-1262.
Thompson JA, Felsen G (2013) Activity in mouse pedunculopontine tegmental nucleus reflects action and outcome in a decision-making task. J Neurophysiol 110:2817-2829.
Thompson RF, Spencer WA (1966) Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychol Rev 73:16-43.
Timmann D, Musso C, Kolb FP, Rijntjes M, Juptner M, Muller SP, Diener HC, Weiller C (1998) Involvement of the human cerebellum during habituation of the acoustic startle response: a PET study. J Neurol Neurosurg Psychiatry 65:771-773.
Typlt M, Mirkowski M, Azzopardi E, Ruth P, Pilz PK, Schmid S (2013) Habituation of reflexive and motivated behavior in mice with deficient BK channel function. Frontiers in integrative neuroscience 7:79.
Ukai M, Kobayashi T, Kameyama T (1994) Characterization of the effects of scopolamine on the habituation of exploratory activity: differential effects of oxotremorine and physostigmine. Gen Pharmacol 25:433-438.
Van Dort CJ, Zachs DP, Kenny JD, Zheng S, Goldblum RR, Gelwan NA, Ramos DM, Nolan MA, Wang K, Weng FJ, Lin Y, Wilson MA, Brown EN (2015) Optogenetic activation of cholinergic neurons in the PPT or LDT induces REM sleep. Proc Natl Acad Sci U S A 112:584-589.
Vincent SR, Satoh K, Armstrong DM, Panula P, Vale W, Fibiger HC (1986) Neuropeptides and Nadph-Diaphorase Activity in the Ascending Cholinergic Reticular System of the Rat. Neuroscience 17:167-&.
Wagner AR (1981) SOP: A model of automatic memory processing in animal behavior. In: Information processing in animals: Memory mechanisms (Spear NEM, R.R., ed), pp 5-47. Hillsdale, NJ: Erlbaum Inc.
Wallace TL, Callahan PM, Tehim A, Bertrand D, Tombaugh G, Wang S, Xie W, Rowe WB, Ong V, Graham E, Terry AV, Jr., Rodefer JS, Herbert B, Murray M, Porter R, Santarelli L, Lowe DA (2011) RG3487, a novel nicotinic alpha7 receptor partial agonist, improves cognition and sensorimotor gating in rodents. The Journal of pharmacology and experimental therapeutics 336:242-253.
Walter BLS, A.G. (2014) Midbrain. In: Encyclopedia of the Neurological Sciences (Aminoff MJD, R.B. , ed), pp 28–33: Elsevier.
Wang HL, Morales M (2009) Pedunculopontine and laterodorsal tegmental nuclei contain distinct populations of cholinergic, glutamatergic and GABAergic neurons in the rat. Eur J Neurosci 29:340-358.
Weber M, Schnitzler HU, Schmid S (2002) Synaptic plasticity in the acoustic startle pathway: the neuronal basis for short-term habituation? Eur J Neurosci 16:1325-1332.

Welker WI (1957) Free Versus Forced Exploration of a Novel Situation by Rats. Psychological Reports 3:95-108.
Williams JM, Hamilton LW, Carlton PL (1975) Ontogenetic dissociation of two classes of habituation. J Comp Physiol Psychol 89:733-737.
Wu MF, Jenden DJ, Fairchild MD, Siegel JM (1993) Cholinergic mechanisms in startle and prepulse inhibition: effects of the false cholinergic precursor n-aminodeanol. Behav Neurosci 107:306-316.
Wynn JK, Dawson ME, Schell AM, McGee M, Salveson D, Green MF (2004) Prepulse facilitation and prepulse inhibition in schizophrenia patients and their unaffected siblings. Biol Psychiatry 55:518-523.
Wynn JK, Green MF, Sprock J, Light GA, Widmark C, Reist C, Erhart S, Marder SR, Mintz J, Braff DL (2007) Effects of olanzapine, risperidone and haloperidol on prepulse inhibition in schizophrenia patients: a double-blind, randomized controlled trial. Schizophr Res 95:134-142.
Xiao C, Cho JR, Zhou CY, Treweek JB, Chan K, McKinney SL, Yang B, Gradinaru V (2016) Cholinergic Mesopontine Signals Govern Locomotion and Reward through Dissociable Midbrain Pathways. Neuron 90:333-347.
Yeomans J (2012) Muscarinic Receptors in Brain Stem and Mesopontine Cholinergic Arousal Functions. In: Muscarinic Receptors (Fryer AD, Christopoulos, A. & Nathanson, n.M., ed), pp 243-263: Springer.
Yeomans JS, Pollard BA (1993) Amygdala efferents mediating electrically evoked startle-like responses and fear potentiation of acoustic startle. Behav Neurosci 107:596-610.
Yeomans JS, Bosch D, Alves n, Daros A, Ure RJ, Schmid S (2010) GABA receptors and prepulse inhibition of acoustic startle in mice and rats. Eur J Neurosci 31:2053-2061.
Young JW, Meves JM, Tarantino IS, Caldwell S, Geyer MA (2011) Delayed procedural learning in alpha7-nicotinic acetylcholine receptor knockout mice. Genes, brain, and behavior 10:720-733.
Zhang J, Forkstam C, Engel JA, Svensson L (2000) Role of dopamine in prepulse inhibition of acoustic startle. Psychopharmacology (Berl) 149:181-188.

2. Chapter 2

VAChT KD Mice Show Normal Prepulse Inhibition but Disrupted Long-term Habituation

Sections 2.1-2.5 were published previously, see Schmid S, Azzopardi E, De Jaeger X, Prado MA, Prado VF (2011) VAChT knock-down mice show normal prepulse inhibition but disrupted long-term habituation. Genes Brain Behav 10:457-464.

2.1 Introduction

The neurotransmitter acetylcholine (ACh) plays an important role in both the central and peripheral nervous system. Disruptions in the central cholinergic system has been associated with different human disorders, including Alzheimer's disease (Felder et al., 2001; Mesulam, 2004), and Schizophrenia (Felder et al., 2001; Barak, 2009). One hallmark in Schizophrenia is impaired prepulse inhibition (PPI) of the acoustic startle response. Prepulse inhibition is a measure of sensorimotor gating, referring to the inhibition of the startle response to a sudden intense auditory stimulus (pulse) because of prior presentation of a sub-threshold stimulus (prepulse). This reflects the ability to suppress sensory information from processing and conscious awareness. It has been used as an assay and endophenotype of sensorimotor gating deficits exhibited by patients with Schizophrenia.

Prepulse inhibition can pharmacologically be disrupted by systemic injections of cholinergic muscarinic antagonists (as reviewed by Jones and Shannon, 2000a; Barak, 2009), whereas systemic nicotine has been shown to enhance PPI in Schizophrenic patients and healthy humans (Kumari et al., 2001, 2002; Postma et al., 2006; Kumari et al., 2008), as well as in different animal models (Acri et al., 1994; Curzon et al., 1994). Prepulse inhibition of the startle response has been proposed to be at least partly mediated by inhibitory cholinergic projections from the laterodorsal tegmental (LDT) and pedunculopontine tegmental nucleus (PPT) to the startle-mediating neurons in the pons (Koch, 1993; Bosch and Schmid, 2006, 2008). Besides its descending inhibition to the caudal pontine reticular nucleus (PnC), the PPT projects to higher brain structures, including the Thalamus, Substantia Nigra and Ventral Tegmental Area (Steriade et al., 1990; Yeomans, 1995; Blaha et al., 1996; Fendt et al., 2001). It is suggested that these

cholinergic PPT projections may be responsible for cortical activation and eliciting approach behaviours, while inhibiting avoidance behaviours like startle through the descending projections (Fendt et al., 2001).

Habituation is another form of sensory filtering that is disrupted in Schizophrenia and delayed in patients suffering from Autism Spectrum Disorders. Short-term habituation (STH) describes the decline of a behavioural response (e.g. startle) to repeated presentation of the same stimulus within a testing session, whereas long-term habituation (LTH) describes the decline of the first (or the average) response over consecutive testing sessions. Habituation is a basic form of non-associative learning. Although STH of startle occurs within the primary startle pathway (Davis et al., 1982a; Schmid et al., 2010), LTH is disrupted by lesions outside of this pathway, for example in the cerebellar vermis (Leaton and Supple, 1986, 1991). Yet, there is little indication for a role of ACh in habituation of startle (Hughes, 1984).

Habituation and PPI deficits have been associated with cognitive symptoms in neural disorders. The cholinergic system plays a major role in cognitive function and drugs facilitating cholinergic transmission have been developed as cognitive enhancers. Efficient synaptic release of ACh depends on its transport into synaptic vesicles by vesicular ACh transporter (VAChT; Prado et al., 2006; de Castro et al., 2009). Homozygous VAChT knock-down mice (VAChT KD^{HOM}) with a 65% reduced immunoreactivity for VAChT in the brain show decreased ability to refill synaptic vesicles (Prado et al., 2006; de Castro et al., 2009). Homozygous VAChT KD mice have a reduced capability to sustain ACh release and the injection of the cholinesterase inhibitor galantamine has previously shown to reverse motor and memory deficits in mutant mice (Prado et al., 2006; de Castro et al., 2009). Homozygous VAChT KD mice are therefore an excellent model to study

the consequences of disruptions in central cholinergic function. We studied the effect of VAChT KD on basic cognitive processes such as STH and LTH as well as PPI of startle.

2.2 Methods

2.2.1 Subjects

For this experiment we used a mutant mouse line which had the vesicular acetylcholine transporter protein knocked-down (VAChT KD^{HOM}). Generation and genotyping of the mice has been described before (Prado et al., 2006; de Castro et al., 2009). The mice were generated by targeting the 5' untranslated region of the VAChT gene for homologous recombination by inserting a TK-Neo resistance cassette 1.5 kb downstream from the VAChT stop codon. The placement of this cassette interrupts VAChT expression in cholinergic neurons of the central nervous system, but leaves somatomotor cholinergic neurons relatively intact. These mice had a mixed 129/terSV x C57BL/6J background. Mice were back-crossed for at least three generations (N3). Ten different breeding pairs of heterozygous mice were bred to generate wild-type (WT) and homozygous KD littermates. Only male WT and KD animals were used in this study. They were housed in groups of 3-4 in a temperature controlled room a with 12 hour light–dark cycle. Food and water were available *ad libitium*. All testing occurred during the light phase while the animals were between 2-5 months of age. Animals were cared for according to the ethical guidelines of the University of Western Ontario Animal Use Subcommittee and Canadian Council on Animal Care (CCAC).

2.2.2 Drugs

In an attempt to rescue cholinergic function, galantamine (1 mg/kg, intraperitoneal (IP)), an acetylcholine-esterase inhibitor, was administered to both WT and KD animals. Galantamine was dissolved in sterile saline (0.9% NaCl) to a dilution of 1 mg/ml. Both genotypes were also given saline as a control. Injections were given either before testing (WT n=14: n=7 saline, n=7 galantamine. KD n=37, n=18 saline, n=19 galantamine) or after (KD saline n=9, galantamine n=10) in order to elucidate the effects of ACh during encoding and retrieval or consolidation processes.

2.2.3 Startle Testing

Mice were randomly assigned to a sound-proofed startle box (Med Associates, St Albans, VT, USA) in which they consistently underwent all behavioural testing. For an overall schematic representation of the behavioural protocol see figure 2.1.

Mice were acclimated to the startle box for 5 minutes/day for 3 days with background noise (65 dB SPL white noise). On the final day of acclimation animals also underwent an input/output (I/O) test. For this test animals are placed in their respective holders and placed in the startle box. Testing began with an acclimation phase (5 min, 65 dB white noise) which was followed by the presentation of white noise bursts (20 ms duration) starting at 65 dB SPL and increasing to 120 dB SPL (increasing by 5 dB SPL with each trial for a total of 12 trials; 15 seconds between trials).

The I/O test allowed us to ensure our animals have normal hearing ability as well as assessing the animal's individual startle reflex magnitude. Rodents fall within a spectrum between high and low startle reactivity much like humans (Hutchison et al., 1997; Schwegler et

al., 1997). The I/O function assessed where the mice fall in this spectrum and according to this we determined a gain setting. This gain amplified the signal from the movement sensitive platform to the digitizer, allowing for a more a more accurate reading. If the signal was too low, the transducer (which converted the amount of movement-induced displacement into an electrical signal) may not have been sensitive enough to detect changes, specifically decreases, in startle magnitude. We increased the output so that each animal responded within the optimal range, making the data more stable between animals and improved measurement accuracy. Using this method we did not have to separate data into low and high startling reactivity as previous studies have done (see Hutchison et al., 1997) which can make analysis complicated. We prescribed high startling mice a gain of 1, medium startling 2-3, and low startling 4 (refer to figure 1.2). This gain was kept constant for each animal over every day of testing. We factored out the gain (by dividing the amplitude of the startle by the gain factor) when analyzing baseline startle amplitudes, to reduce confounding of results.

Once I/O testing was completed, the animals began the experimental protocol. Animals were tested once daily for 5 days. The protocol consisted of a 5 minute acclimation phase (65 dB SPL white noise) and two blocks of trials (see figure 2.1). Block one was used to assess habituation and block two was used to assess PPI.

The first block consisted of 30 pulse-alone trials. A startle pulse was a 105 dB SPL (20 ms duration) burst of white noise. The intertrial interval (ITI) was 15 sec. This pulse was chosen based on data from the I/O test as it was the first to induce the maximal startle amplitude (see figure 2.2). Using a higher startle pulse could risk hearing damage. Following the pulse the resulting startle magnitude was recorded digitally using Med Associate software. The magnitude reflected

the amount of displacement, induced by the startle, of the movement sensitive platform placed at the bottom of the startle box.

The second block of trials was a mix of 60 prepulse and 10 pulse-alone trials. The pulse-alone trials, which were exactly the same as described in block one, measured baseline startle magnitude. The prepulse trials consisted of a 85 dB SPL (4 ms duration) prepulse with an interstimulus interval (ISI) of either 6, 12, 50, 100, 200 or 250 ms. This created 6 types of prepulse trials, and 10 trials of each type. All trials were presented in a pseudo-randomized manner to ensure an accurate baseline startle measurement. Startle magnitudes, recorded digitally in the same manner as described previously, were compared between pulse-alone trials and prepulse trials.

When galantamine (1 mg/kg, intraperitoneal) was administered either pre or post testing (see figure 2.1), mice only completed block one of testing as only habituation was necessary. Presenting a stimulus too many times can cause sensitization to that stimulus, which would confound our results (Plappert et al., 1999). Therefore for this part of the study, all long-term habituation results were derived during a separate testing session where just the acclimation phase and block one were presented. If an animal was re-used for injection testing, sessions were separated by a minimum of 2 weeks.

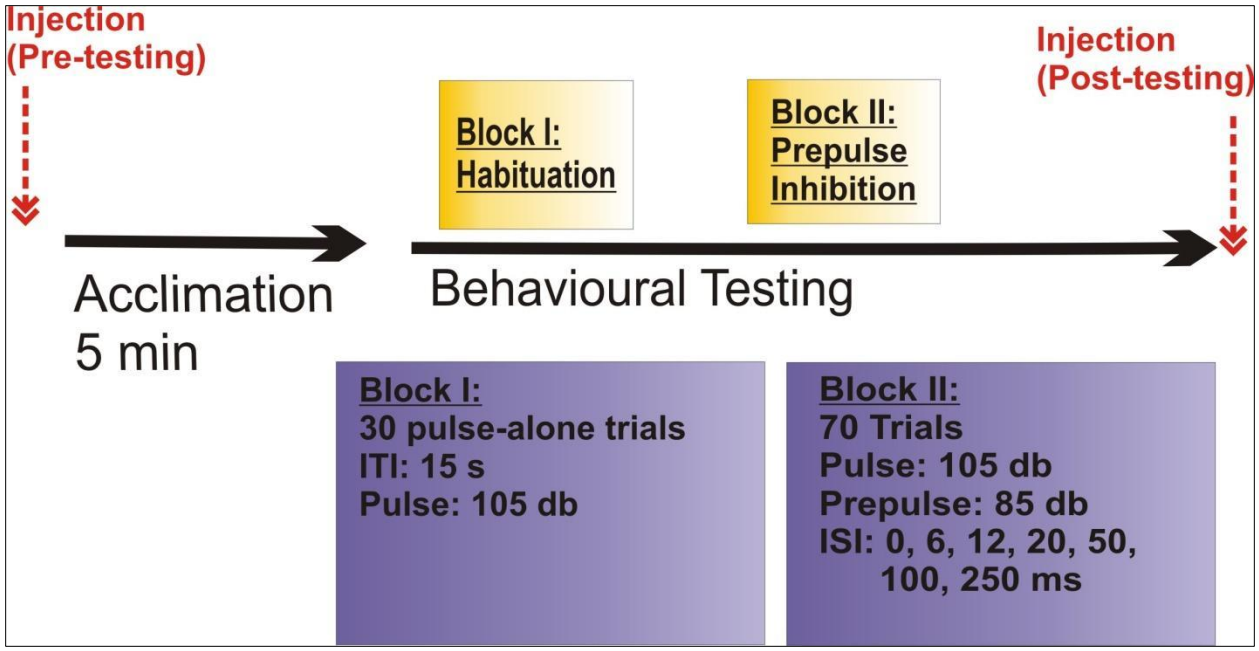

Figure 2.1 A Graphic Representation of Sensory Filtering and Sensorimotor Gating Testing
This represents one day of testing. This testing is repeated for every day, for 5 days. Block I assesses habituation, and block II assesses PPI. All trials in block II appear in a pseudo-randomized order. Only the habituation rescue experiments contained injections, which occurred either directly before or directly after testing (Intertrial interval: ITI, interstimulus interval: ISI).

2.2.3.1 Data Analysis for Startle Testing

To analyze PPI and habituation data, unpaired or paired Student's t tests or two-way repeated measures analysis of variance (ANOVAs) were used. To assess if VAChT KD^{HOM} animals displayed differences in STH, all responses from block one were normalized by dividing them by the average of the first three responses. We analyzed this using a two way ANOVA (trial number x genotype). This was done for everyday of testing and then averaged across days. For LTH the first three trials of block one were averaged for each day of testing. These averages were combined across animals and then plotted (across days) for visualization. We also normalized all

data points to the average of the first 3 trials on day 1, and plotted values across days. To statistically analyse long-term habituation (LTH) we used a two-way ANOVA (day x genotype). For rescue experiments, for each genotype, we analyzed LTH using a two-way ANOVA (day x drug type).

In our studies, PPI was expressed as percent of baseline startle. This means that the responses from the prepulse trials of block two were divided by the average of the pulse alone trials of block two (baseline startle). This was then multiplied by 100 to give a percent ([startle/baseline startle] x 100). We determined percent of startle for each trial type, for each day. We then performed a two-way ANOVA (genotype x day) to determine if PPI improved across days of testing. We then averaged PPI values across days and performed a two way ANOVA (trial type x genotype).

We assessed baseline startle, to ensure startle ability between genotypes was equal, by using the pulse-alone trials of block two. This was the optimal measurement as startle responses within block one are not stable due to habituation. Criteria for significance for all statistical tests was $p<0.05$.

2.3 Results

2.3.1 Both Genotypes Have the Same Startle Reactivity

Fourteen WT and 12 VAChT KD^{HOM} mice were tested for their startle response amplitude with increasing startle stimulus intensities from 65 to 120 dB SPL white noise in 5 dB SPL steps. All startle boxes were calibrated to box 1 in order to allow for a direct comparison of startle amplitudes. As shown in figure 2.2a,b, the absolute startle response amplitude differed

substantially between individual mice with high, intermediate and low startling mice in both genotypes. A two-way repeated measures ANOVA (genotype x sound intensity) showed no effect of the genotype on startle response amplitudes ($F_{(1,311)}$= 0.08, p=0.78), however there was a significant effect of the sound intensity ($F_{(11,301)}$= 17.21, p<0.0001), but no interaction of the two factors ($F_{(1,311)}$= 0.9, p=0.33). Although high startling mice overshot the scale, low startling mice barely raised the signal above the noise level, making it difficult to quantify startle attenuation by PPI or habituation, which illustrates the necessity of gains for appropriate startle magnitude detection. The average startle amplitude of the final three startle trials from this test was also used to further compare absolute startle responses amplitudes between genotypes. A two-tailed independent Student's t-test indicated no difference in baseline startle magnitude amplitudes between genotypes (t_{24}=0.7, p>0.05).

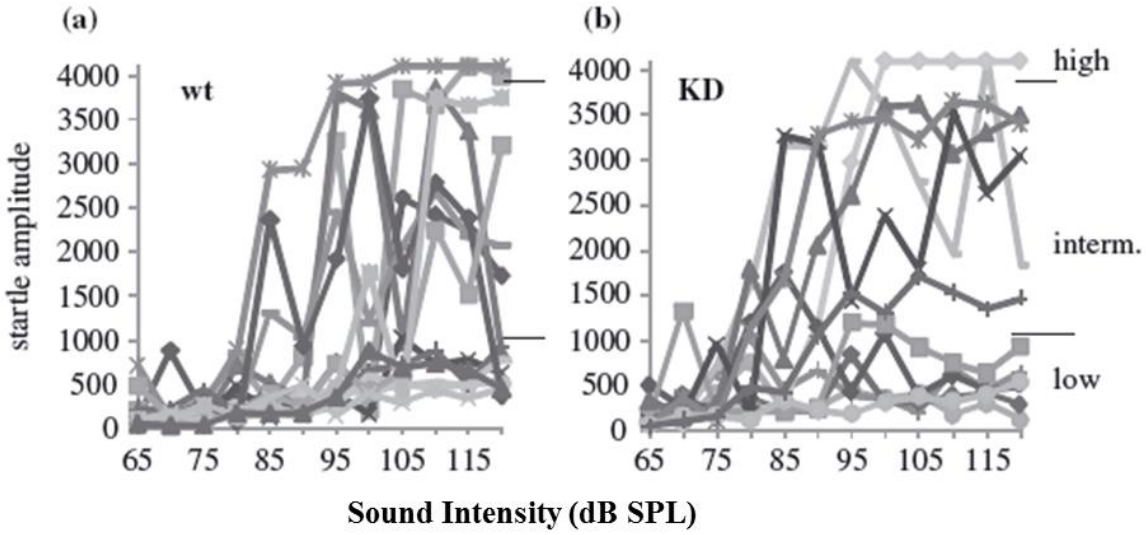

Figure 2.2 Individual Input/Output Functions of VAChT KD^{HOM} Mice Compared to Wild-type

After three acclimation sessions, animals were exposed to 65 dB SPL background noise and increasing startle stimulus intensities from 65 to 120 dB SPL. No difference in startle response amplitudes between genotypes was observed. Both WT (a) and KD mice (b) reached maximum startle amplitude at around 105 dB SPL; however, there was considerable variability in the maximum startle responses between individuals within both the groups. Although some mice overshot our range of measurement, others barely raised the signal above noise level. In subsequent experiments, gain factors for the measured motor response were adjusted according to the following scheme: mice that overshot were measured with a gain of 1.0, intermediate mice were measured with the default gain of 2 and low startling mice were measured with a gain of 4.

2.3.2 Prepulse Inhibition of the Acoustic Startle Response is Normal in VAChT KD^{HOM} Mice

Prepulse inhibition of startle was tested across ISIs. As shown in figure 2.3, VAChT KD^{HOM} mice and their WT littermates did not differ in their ability to suppress startle stimuli that were preceded by a prepulse as we could detect no influence of genotype ($F_{(1,58)}$= 0.82, p=0.78). Prepulse inhibition differed across ISIs ($F_{(5,58)}$=11.5, p<0.001), but this was unaffected by genotype as there was no interaction between genotype and ISI ($F_{(5,58)}$=0.8, p=0.84).

Both groups inhibited their startle by around 70% at ISIs between 5 and 50 ms, with a maximum PPI of 75% at 50 ms ISI. Prepulse inhibition slightly decreased in both the groups at ISIs of 100 ms (66% for WT and 67% for KD) and 250 ms (50% WT and 54% KD). Overall, this suggests that PPI is unaffected by the knockdown of VAChT.

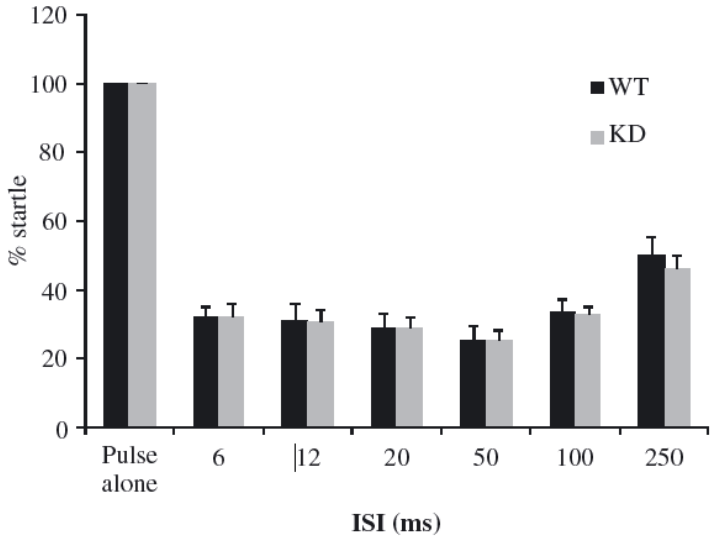

Figure 2.3 Prepulse Inhibition is Unaltered by VAChT Knock Down

Startle response amplitudes to acoustic startle stimulus alone trials were compared with startle amplitudes when startle stimuli were preceded by an acoustic prepulse. This prepulse occurred at different interstimulus time intervals (ISI), as indicated. The average startle amplitude of each animal to startle stimulus alone trials was set to 100% and all other responses are expressed as percentage of it. Both genotypes, the VAChT KD^{HOM} mice (KD) as well as their WT littermates, showed up to a 75% reduction of startle by the prepulse with no difference between groups (n=16/genotype).

2.3.3 Short-Term Habituation is Unaffected by VAChT KD, but Long-Term is Impaired

All animals were habituated to startle stimuli during block I prior to testing PPI. As shown in figure 2.4a, both groups of animals habituated to 70–75% of their initial startle amplitude (measured as the average of the first two responses) within each testing session. An ANOVA showed a significant main effect of trial number ($F_{(29,270)}$= 2.98, p<0.001), with no interaction of genotype and trial number ($F_{(20,270)}$= 0.75, p=0.82), which confirmed no effect of genotype on STH.

We tested for LTH by analyzing the average of the first three startle responses over the 5 days of testing. As shown in figure 2.4b initial startle responses declined over the course of 5 days to around half of the amplitudes on day 1 in WT mice. In contrast, VAChT KD^{HOM} mice did not show any LTH across sessions. The ANOVA showed a significant interaction between the factors genotype and day ($F_{(4,145)}$= 2.52, p=0.045).This effect is summarized in figure 2.4c. This figure displays the course of both STH and LTH over five consecutive days of testing in both genotypes. Both genotypes still showed STH, but only the WT mice showed LTH as the mutant mice startled to the same extent at the beginning of each test session.

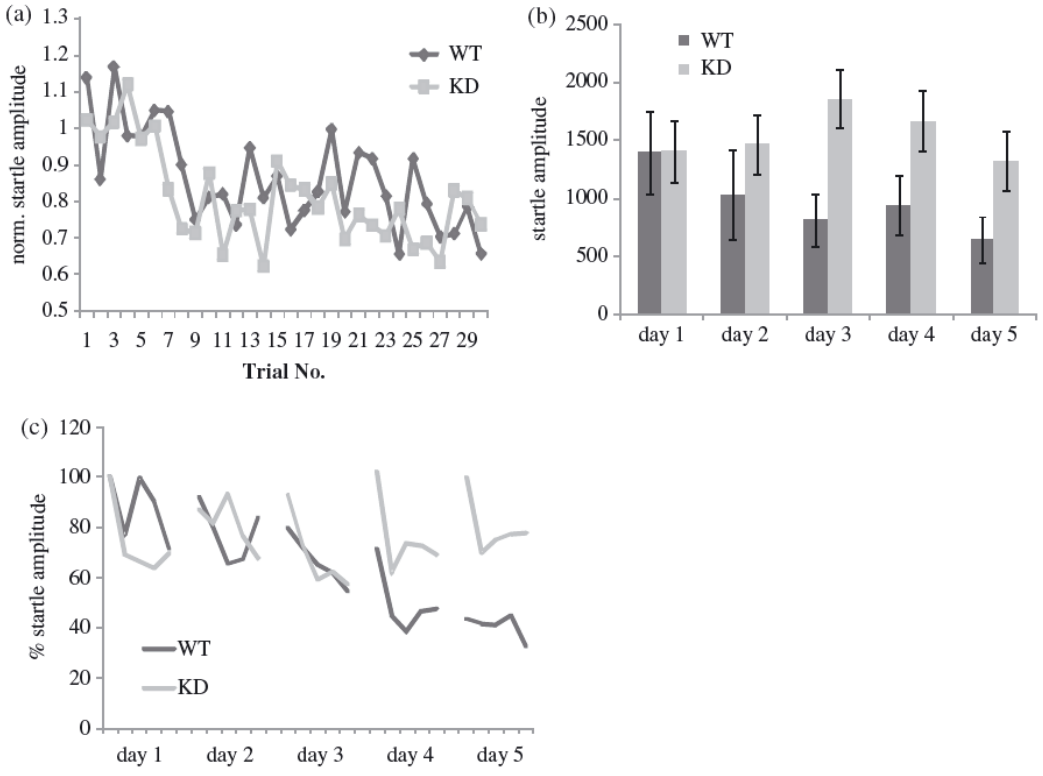

Figure 2.4 Short-Term Habituation is Normal in VAChT KD^{HOM} Mice, but Long-Term is Impaired
(a) Short-term habituation in VAChT KD^{HOM} mice was similar to that of their WT littermates. The startle response amplitude of each mouse was normalized to the average of its first three startle responses. (b) The long-term habituation of VAChT KD^{HOM} and WT mice. The averages of the first three startle responses of each day were calculated for each mouse and then averaged within genotypes. Startle response magnitude declined over days in WT animals, but not in VAChT KD mice. (c) The course of STH and LTH over 5 days. For more clarity, six consecutive startle responses of an animal were always averaged (blocks of six, for a total of five blocks). The data of each animal was then normalized to the value of the first block (=100%). The graph shows that both genotypes show STH at every day, but whereas the overall startle amplitudes decline in WT animals over the 5 days, startle amplitudes remain at the same level in VAChT KD^{HOM} mice, indicating a lack of LTH (n=16/genotype).

2.3.4 Rescuing the Long-Term Habituation Deficit

Next, we tested if we could rescue LTH by increasing cholinergic tone using galantamine injections (1 mg/kg IP) prior to experiments. For this experiment, half of the WT and VAChT KD^{HOM} mice were injected with saline and half with galantamine before the gain setting I/O test (WT n=14, VAChT KD^{HOM} n=37). A two-way ANOVA (genotype x drug) was performed on the average of the final three startle responses of the gain setting test (equal gains for all groups). It did not detect any difference between genotypes ($F_{(1,13)}$= 0.03, p=0.86) or drug ($F_{(1,13)}$= 3.93, p=0.06) or interaction between these factors ($F_{(1,13)}$= 2.74, p=0.11). In order to further exclude the possibility that galantamine injections caused a general increase of startle responses, 16 WT and 16 VAChTKD^{HOM} mice were injected with saline after 2 days acclimation to the boxes and startle was tested. On the next day, they received a galantamine injection prior to testing startle amplitude (figure 5a). A paired t-test showed no difference in baseline startle amplitudes between saline and galantamine injections in WT (t_{15}=1.2, p=0.12) or VAChTKD^{HOM} mice (t_{15}=0.9, p=0.19).

For the rescue experiment, we injected animals with galanthamine or saline prior to testing for 5 days. In WT animals, a repeated measure ANOVA (injection x trial OR day) showed that there was no effect of injection group on STH. We found no interaction between drug and trial ($F_{(29,390)}$= 1.08, p=0.35, figure 2.5b), and no effect of repeated galantamine injection over 5 days on LTH ($F_{(4,66)}$= 1.54, p=0.2, figure 2.5c). We noted that both injection groups display less STH and LTH than in the previous experiment without injections. This is probably due to the aversive nature of the injection procedure itself (compare figure 5b,c with figure 4a–c).

In VAChT KD^{HOM} mice, a repeated measure ANOVA (injection x trial OR day) showed there was also no effect of injection on STH ($F_{(29,270)}$= 0.70, p=0.87, figure 2.5b). However, pre-testing

galantamine-injected VAChTKD^{HOM} mice displayed a decline of their average startle responses between days, whereas saline-injected animals did not show any LTH, confirming the lack of LTH in VAChTKD^{HOM} mice in a second cohort of mice (figure 2.5d). The ANOVA confirmed this as there was a trend towards a significant interaction between drug and day ($F_{(4,22)}$= 1.33, p=0.054). Unpaired Student's t-tests confirmed no difference between injection groups at days 1 (t_{35}=0.9, p=0.39), 2 (t_{35}=1.75, p=0.08) and 4 (t_{35}=1.68, p=0.08), but a significant difference between injection groups at days 3 (t_{35}=2.6, p=0.01) and 5 (t_{35}=2.4, p=0.02).

In order to test whether the acetylcholine-esterase inhibitor had to be present during learning/retrieval, or if it was sufficient when present during the consolidation phase following learning, we repeated the rescue experiment with the VAChT KD^{HOM} mice, but administered galantamine (or saline) immediately after the behavioural testing. As expected, STH in this group of animals was not affected by the type of injections and animals showed STH comparable to non-injected animals (figure 2.6a, compare figure 2.4a). As revealed by a repeated measures ANOVA (day x drug) galantamine failed to rescue LTH when injected immediately after the behavioural testing session ($F_{(1,159)}$= 0.43, p=0.79), as shown in figure 2.6b,c.

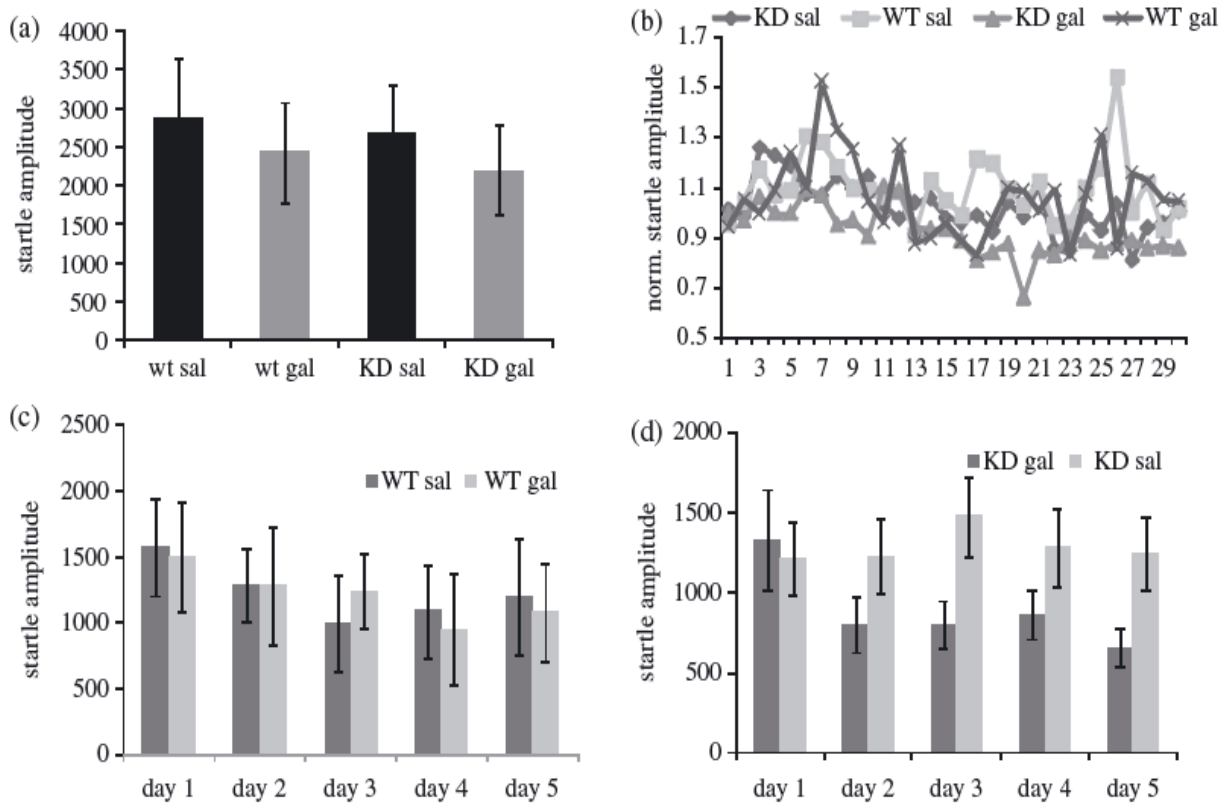

Figure 2.5 Rescue of LTH by Pre-Test Injections of Galantamine

(a) Control experiments were performed which tested the effect of galantamine (gal) vs. saline (sal) on baseline startle responses. Sixteen WT and 16 VAChT KD^{HOM} mice were exposed to acclimation and block I (habituation) in two subsequent days, with sal injection on day 1 and gal injection on day 2. The first three startle responses of each animal after saline were compared with the first three responses after galantamine on the subsequent day. There was no effect of drug type on baseline startle amplitude in either genotype. (b) The STH to 30 startle stimuli in WT (n=23) and VAChT KD^{HOM} mice (n=27) injected with gal or sal. For each mouse, the startle response amplitude of each trial was normalized to the average of its first three startle responses. Short-term habituation was overlaid by initial sensitization in both genotypes (compare with figure 4a), probably due to the aversive nature of injections. No difference between genotypes or injection group was detected. (c) The LTH of WT mice following 5 days of sal or gal treatment prior to behavioural testing. The average of the first three startle amplitudes of each day were

calculated for each mouse and then averaged within treatment. No differences between injection groups could be detected at any of the 5 days (n=7/group). Finally, (d) displays the LTH of VAChT KD^{HOM} mice following 5 days of sal or gal injections prior to behavioural testing. Homozygous VAChT KD mice that were injected with gal (n=19) show a decline of their startle responses mainly after day 1. Animals injected with sal (n=18) did not show LTH, confirming the previous results.

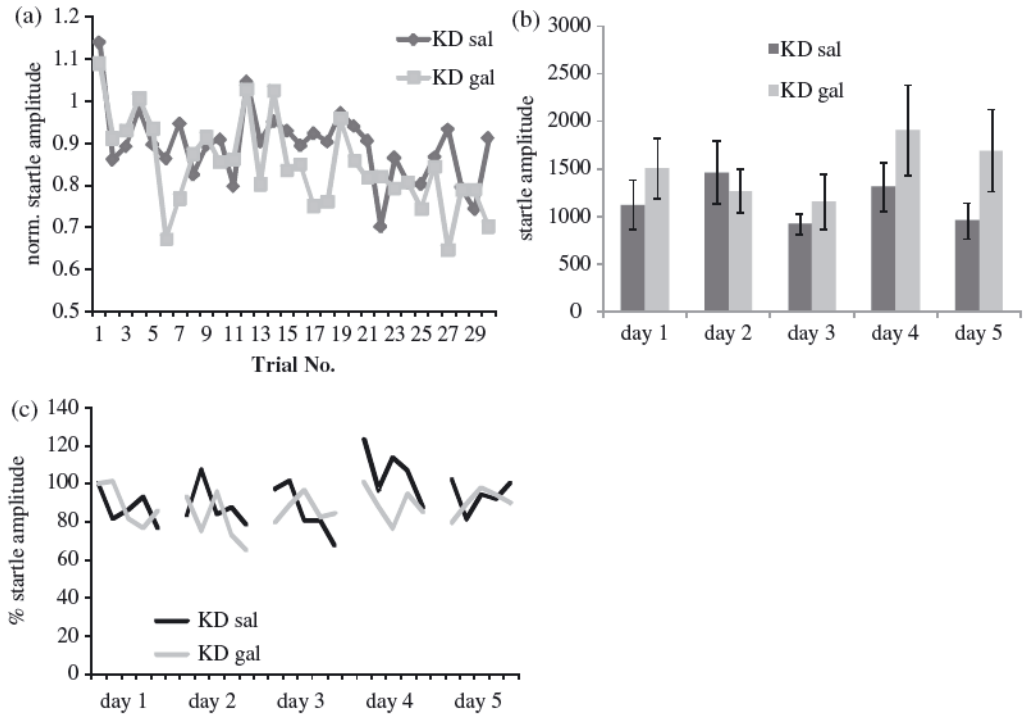

Figure 2.6 Post-Test Injections of Galantamine Did Not Rescue LTH

Animals were injected with 1 mg/kg of galantamine (gal) immediately after behavioural testing. (a) The STH to 30 startle stimuli in VAChT KD^{HOM} mice injected by gal and in control VAChT KD^{HOM} mice injected by saline (sal). For each mouse, the startle responses for all 5 days were normalized to the average of its first two startle responses. Both injection groups show intact STH. (b) The LTH of sal- and gal-injected mutant mice. The averages of the first three startle responses of each day were calculated for each mouse and averaged within treatment. Both groups of animals show the same level of startle amplitudes over the 5 days. (c) The course of STH and LTH over 5 days. For more clarity, six consecutive startle responses of an animal were averaged (blocks of six, for a total of 5 blocks). The data of each animal was then normalized to the value of the first block (=100%). Both groups of animals show STH, but no LTH, over 5 days (KD^{HOM} mice sal n=9, KD^{HOM} mice gal n=10).

2.4 Discussion

Homozygous VAChT knock-down mice had a 65% reduced expression of the VAChT protein resulting in reduced cholinergic neurotransmission. It has been shown previously that these mice have a decreased ability to maintain physical activity. This impairment was most evident during tasks where prolonged muscle contraction was required, such as in the rotarod or wire hang tests. Heterozygous VAChT KD^{HOM} mice with a 40% reduction of VAChT immunoreactivity display slower motor learning than their WT controls and impaired object and social recognition, however their motor function, olfaction and spatial memory are mainly unperturbed (Prado et al., 2006; de Castro et al., 2009). In this study, we found that VAChT KD^{HOM} mice have normal PPI and STH of startle, but disrupted LTH. Surprisingly, our data indicates that VAChT KD^{HOM} mice do not exhibit lower startle responses than their WT littermates, as assessed by comparison of the I/O function (figure 2.2). Small differences between genotypes, however, would be very difficult to detect, given the huge variability of startle responses between animals of the same genotype. It still seems safe to state that the previously reported motor deficits of the VAChT KD^{HOM} mice did not have a major impact on our results. A short startle response every 15-20 seconds may not be sufficient to deplete cholinergic synaptic vesicles. Indeed, the previously reported motor deficits were specific for prolonged motor activity, whereas initial synaptic release and release probability have been shown to be normal in these mice (Prado et al., 2006).

2.4.1 Prepulse inhibition

The inhibitory effect of a prepulse on the startle reflex is largely assumed to occur via cholinergic PPT projections to the PnC, but descending limbic cortico–striato–pallido–pontine

circuitry is also known to regulate pontine inhibitory tone. This regulation determines the degree to which the prepulse can inhibit the subsequent startle response. The cholinergic system closely interacts with dopaminergic systems in the striatum; therefore, it is not clear to what extent cholinergic drugs affect the PPI circuitry directly as opposed to PPI modulation through alterations in the signaling of other neurotransmitter systems, including dopamine. The expression of normal PPI in VAChT KD^{HOM} mice was surprising given the large body of evidence for an important role of cholinergic modulation of PPI and the reported effects of systemic cholinergic drugs.

We tested PPI at different ISIs between prepulse and startle stimuli, since it has been hypothesized that different neurotransmitter systems mediate PPI at different time scales (Jones and Shannon, 2000a, b; Fendt et al., 2001; Jones et al., 2005; Yeomans et al., 2010). We chose the prepulse intensity that yielded maximum PPI without causing a startle reaction itself (85 dB SPL). We varied the ISI from the commonly used 100 ms to 30 and 50 ms, which are the ISIs yielding maximum PPI in mice, plus a very short and a long ISI, in order to ensure that we did not miss any cholinergic contribution. There was no PPI deficiency at any ISI. However, as with the lack of an effect on baseline startle reactivity, we cannot exclude the possibility that although cholinergic transmission was reduced in the mutant mice, it still may have been sufficient for PPI signaling in response to a short prepulse every 15-20 seconds. Furthermore, other neurotransmitter systems contributing to PPI, such as GABA (Kodsi and Swerdlow, 1995; Fendt, 1999; Yeomans et al., 2010), could be upregulated and compensate for the lack of cholinergic transmission in these mice. The unperturbed PPI in the mutant mice therefore cannot lead to the assumption that ACh plays no role in mediating and modulating PPI.

2.4.2 Habituation

Short-term habituation of startle is assumed to occur within the glutamatergic primary startle pathway. More specifically, the glutamatergic synaptic terminals of sensory afferent fibers projecting on the startle-mediating giant neurons in the PnC are assumed to undergo synaptic depression during STH (Weber et al., 2002; Simons-Weidenmaier et al., 2006; Schmid et al., 2010). The role of cholinergic modulation in habituation has been investigated in the past and it is assumed that STH to external stimuli is not directly modulated by cholinergic neurotransmission (Hughes, 1984), which is in accordance with our results. The disruption of STH in both treatment groups in the rescue experiment with pre-test injections is likely because of the aversive procedure of IP injections immediately prior to behavioural testing. Aversive stimuli can cause sensitization and fear-potentiation of startle that could override the habituation process (Groves and Thompson, 1970). The fact that STH was normal in both groups in the post-test injection rescue experiment supports this conclusion.

Galantamine injections did not enhance baseline startle responses. Although it prolongs the effect of ACh, it might not affect the initial startle response amplitude, but rather the duration. Most importantly, pre-test galantamine injections seem to restore LTH in VAChT KD^{HOM} mice, although the ANOVA failed to show a clear significance with *p* values just slightly above 0.05. A paired t-test confirmed a significant difference in startle amplitudes between galantamine- and saline-injected VAChT KD^{HOM} mice for days 3 and 5. The rescue experiment was difficult to perform, as the injection procedure itself leads to sensitization, which opposed habituation in the following startle test (compare STH and LTH in figures 4 and 5), and decreasing the differences between WT and VAChTKD^{HOM} mice in terms of habituation. Disruptions in LTH

could be rescued only by pre-test injections of galantamine, when galantamine was present during acquisition and expression of LTH. Unfortunately, unlike in many other learning paradigms, acquisition and expression of learning are difficult to separate in LTH experiments. Galantamine did not restore LTH when present during the consolidation phase only (post-test injections).

Little is known about mechanisms underlying LTH of startle. Long-term habituation can be disrupted by lesions of the cerebellar vermis (Leaton and Supple, 1986, 1991). Thus it is an extrinsic modulation of startle that employs cellular substrates different from the ones that mediate startle and STH. There is evidence for a cholinergic innervation of the cerebellum (Jaarsma et al., 1997), but its connection to the vermis or to LTH is unclear. Although the cerebellar vermis is important for LTH, the cholinergic PPT projections to the PnC might be a downstream effector mediating the inhibition of startle during LTH. An increase of the cholinergic tone in this projection could mediate LTH. Interestingly, the first injection of galantamine seemed to induce maximum LTH, as opposed to the gradual startle decline during normal LTH. This would be expected if galantamine strongly enhanced cholinergic activity as opposed to a gradual increase in cholinergic tone during LTH. A tonic cholinergic function would also be more vulnerable to reduced cholinergic neurotransmission in VAChT KD^{HOM} mice, as opposed to a transient cholinergic activation during PPI.

Interestingly, the lack of LTH to startle stimuli in VAChTKD^{HOM} mice is paralleled by a lack of habituation to a juvenile intruder in these mice (Prado et al., 2006). Moreover, mutant mice showed a deficit in object recognition, which could also be explained by a lack of habituation to the recently explored object. It will be crucial to gain more information about the respective

underlying circuitry and mechanisms in order to elucidate in what way these disruptions are connected to each other. Future studies should seek to include comparisons of reflexive behaviour, like startle, versus non reflexive behaviours like locomotion and exploratory behaviour.

In conclusion, although PPI and STH were not impaired, the unexpected disruption of LTH in VAChT KD^{HOM} mice gives us valuable insights into both cognitive functions of cholinergic neurotransmission and mechanisms underlying LTH of startle. Future experiments may want to address whether the LTH deficit is specifically mediated by the cholinergic dysfunction in the midbrain (i.e. PPT) or by higher brain areas modulated by the basal forebrain cholinergic cell groups, using brain region selective regional knockouts for VAChT.

2.5 References

Acri JB, Morse DE, Popke EJ, Grunberg NE (1994) Nicotine increases sensory gating measured as inhibition of the acoustic startle reflex in rats. Psychopharmacology (Berl) 114:369-374.

Barak S (2009) Modeling cholinergic aspects of schizophrenia: focus on the antimuscarinic syndrome. Behav Brain Res 204:335-351.

Blaha CD, Allen LF, Das S, Inglis WL, Latimer MP, Vincent SR, Winn P (1996) Modulation of dopamine efflux in the nucleus accumbens after cholinergic stimulation of the ventral tegmental area in intact, pedunculopontine tegmental nucleus-lesioned, and laterodorsal tegmental nucleus-lesioned rats. J Neurosci 16:714-722.

Bosch D, Schmid S (2006) Activation of muscarinic cholinergic receptors inhibits giant neurones in the caudal pontine reticular nucleus. Eur J Neurosci 24:1967-1975.

Bosch D, Schmid S (2008) Cholinergic mechanism underlying prepulse inhibition of the startle response in rats. Neuroscience 155:326-335.

Curzon P, Kim DJ, Decker MW (1994) Effect of nicotine, lobeline, and mecamylamine on sensory gating in the rat. Pharmacol Biochem Behav 49:877-882.

Davis M, Gendelman DS, Tischler MD, Gendelman PM (1982) A primary acoustic startle circuit: lesion and stimulation studies. J Neurosci 2:791-805.

de Castro BM, Pereira GS, Magalhaes V, Rossato JI, De Jaeger X, Martins-Silva C, Leles B, Lima P, Gomez MV, Gainetdinov RR, Caron MG, Izquierdo I, Cammarota M, Prado VF, Prado MA (2009) Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. Genes Brain Behav 8:23-35.

Felder CC, Porter AC, Skillman TL, Zhang L, Bymaster FP, Nathanson NM, Hamilton SE, Gomeza J, Wess J, McKinzie DL (2001) Elucidating the role of muscarinic receptors in psychosis. Life Sci 68:2605-2613.

Fendt M (1999) Enhancement of prepulse inhibition after blockade of GABA activity within the superior colliculus. Brain Res 833:81-85.

Fendt M, Li L, Yeomans JS (2001) Brain stem circuits mediating prepulse inhibition of the startle reflex. Psychopharmacology (Berl) 156:216-224.

Groves PM, Thompson RF (1970) Habituation: a dual-process theory. Psychol Rev 77:419-450.

Hughes RN (1984) Cholinergic and monoaminergic substrates of startle habituation. Life Sci 34:2101-2105.

Hutchison KE, Rohsenow D, Monti P, Palfai T, Swift R (1997) Prepulse inhibition of the startle reflex: preliminary study of the effects of a low dose of alcohol in humans. Alcohol Clin Exp Res 21:1312-1319.

Jaarsma D, Ruigrok TJ, Caffe R, Cozzari C, Levey AI, Mugnaini E, Voogd J (1997) Cholinergic innervation and receptors in the cerebellum. Prog Brain Res 114:67-96.

Jones CK, Shannon HE (2000a) Muscarinic cholinergic modulation of prepulse inhibition of the acoustic startle reflex. J Pharmacol Exp Ther 294:1017-1023.

Jones CK, Shannon HE (2000b) Effects of scopolamine in comparison with apomorphine and phencyclidine on prepulse inhibition in rats. Eur J Pharmacol 391:105-112.

Jones CK, Eberle EL, Peters SC, Monn JA, Shannon HE (2005) Analgesic effects of the selective group II (mGlu2/3) metabotropic glutamate receptor agonists LY379268 and LY389795

in persistent and inflammatory pain models after acute and repeated dosing. Neuropharmacology 49 Suppl 1:206-218.
Koch M (1993) Microinjections of the metabotropic glutamate receptor agonist, trans-(+/-)-1-amino-cyclopentane-1,3-dicarboxylate (trans-ACPD) into the amygdala increase the acoustic startle response of rats. Brain Res 629:176-179.
Kodsi MH, Swerdlow NR (1995) Ventral pallidal GABA-A receptors regulate prepulse inhibition of acoustic startle. Brain Res 684:26-35.
Kumari V, Soni W, Sharma T (2001) Influence of cigarette smoking on prepulse inhibition of the acoustic startle response in schizophrenia. Hum Psychopharmacol 16:321-326.
Kumari V, Soni W, Sharma T (2002) Prepulse inhibition of the startle response in risperidone-treated patients: comparison with typical antipsychotics. Schizophr Res 55:139-146.
Kumari V, Antonova E, Geyer MA (2008) Prepulse inhibition and "psychosis-proneness" in healthy individuals: An fMRI study. Eur Psychiatry.
Leaton RN, Supple WF, Jr. (1986) Cerebellar vermis: essential for long-term habituation of the acoustic startle response. Science 232:513-515.
Leaton RN, Supple WF, Jr. (1991) Medial cerebellum and long-term habituation of acoustic startle in rats. Behav Neurosci 105:804-816.
Mesulam M (2004) The cholinergic lesion of Alzheimer's disease: pivotal factor or side show? Learn Mem 11:43-49.
Plappert CF, Pilz PK, Schnitzler HU (1999) Interaction between acoustic and electric sensitization of the acoustic startle response in rats. Behav Brain Res 103:195-201.
Postma P, Gray JA, Sharma T, Geyer M, Mehrotra R, Das M, Zachariah E, Hines M, Williams SC, Kumari V (2006) A behavioural and functional neuroimaging investigation into the effects of nicotine on sensorimotor gating in healthy subjects and persons with schizophrenia. Psychopharmacology (Berl) 184:589-599.
Prado VF et al. (2006) Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron 51:601-612.
Schmid S, Brown T, Simons-Weidenmaier n, Weber M, Fendt M (2010) Group III metabotropic glutamate receptors inhibit startle-mediating giant neurons in the caudal pontine reticular nucleus but do not mediate synaptic depression/short-term habituation of startle. J Neurosci 30:10422-10430.
Schwegler H, Pilz PK, Koch M, Fendt M, Linke R, Driscoll P (1997) The acoustic startle response in inbred Roman high- and low-avoidance rats. Behav Genet 27:579-582.
Simons-Weidenmaier NS, Weber M, Plappert CF, Pilz PK, Schmid S (2006) Synaptic depression and short-term habituation are located in the sensory part of the mammalian startle pathway. BMC Neurosci 7:38.
Steriade M, Datta S, Pare D, Oakson G, Curro Dossi RC (1990) Neuronal activities in brain-stem cholinergic nuclei related to tonic activation processes in thalamocortical systems. J Neurosci 10:2541-2559.
Weber M, Schnitzler HU, Schmid S (2002) Synaptic plasticity in the acoustic startle pathway: the neuronal basis for short-term habituation? Eur J Neurosci 16:1325-1332.
Yeomans JS (1995) Role of tegmental cholinergic neurons in dopaminergic activation, antimuscarinic psychosis and schizophrenia. Neuropsychopharmacology 12:3-16.

Yeomans JS, Bosch D, Alves n, Daros A, Ure RJ, Schmid S (2010) GABA receptors and prepulse inhibition of acoustic startle in mice and rats. Eur J Neurosci 31:2053-2061.

2.6 Link between Chapter 2 and Chapter 3

To re-define the role of ACh in sensory filtering and sensorimotor gating of the ASR, we used a transgenic mouse model with a 65% reduction in VAChT (which loads ACh into secretory vesicles). In these mice secretory vesicles are not re-filled efficiently and therefore the sustained release of ACh is reduced. Using these mice, we were able to discover the important and novel role of ACh in the mediation of long-term habituation which previous studies were unable to determine, mainly due to the methodological considerations. The transgenic mice had normal PPI, which was surprising given the litany of pharmacological studies linking ACh to PPI. Therefore, we suggested that transient release may be relatively intact in this transgenic mouse model, and that this type of cholinergic transmission is critical for PPI; whereas prolonged release mediates LTH.

Our next step was to uncover what cholinergic receptor is involved in the mediation of LTH and PPI. As our observed LTH deficit was rescued by galanthamine, a cholinergic agonist and positive allosteric modulator of nicotinic receptors, we specifically chose to investigate the role of the α7-nAChR. This receptor has also been linked to gating deficits in schizophrenic populations, is abundant within several areas of the sensory filtering and sensorimotor gating pathways, and has been strongly implicated in learning and memory.

3. Chapter 3

Sensorimotor Gating and Spatial Learning in α7-Nicotinic Receptor Knockout Mice

Sections 3.1-3.5 were published previously, see: Azzopardi, E., Typlt, M., Jenkins, B. & Schmid, S. (2013). Sensorimotor gating and spatial learning in α7-nicotinic receptor knockout mice. Genes, Brain and Behavior, 12: 414–423.

3.1 Introduction

Sensorimotor gating refers to the ability of the brain to implicitly filter unnecessary sensory information, preserving its limited neuronal capacity for the processing of salient information. Prepulse inhibition (PPI) and habituation of the acoustic startle response represent two different behavioural measures of sensory filtering (Braff et al., 1978). Prepulse inhibition (PPI) occurs when the presentation of a sensory stimulus (prepulse) reduces the behavioural response to a strong startling stimulus (pulse). Theoretical expositions suggest that the processing of the prepulse actively inhibits the processing of the pulse, resulting in decreased responsiveness (for review see Koch et al., 1999). The startle response can also be used to assess habituation of a reflexive behaviour. Habituation is defined as the progressive decrease in response amplitude following repeated exposure to the stimulus. There are two forms of habituation: short-term and long-term, which refer to the attenuation of responding within a testing session or across multiple testing sessions, respectively.

Nicotine is well known to enhance PPI (Acri, 1994; Acri et al., 1994; Faraday et al., 1999; Ingram et al., 2005), but the responsible nicotinic receptor subtype is unknown. Pharmacological studies have suggested a role of α7-nicotinic acetylcholine receptors (nAChRs). Positive modulation of α7-nAChRs improves auditory gating in the DBA/2 mouse model of Schizophrenia (Simosky et al., 2001), and rescues apomorphine and MK801-induced PPI deficits in rats (Dunlop et al., 2009; Wallace et al., 2011). Surprisingly, previous studies using α7-nAChR knockout (KO) mice have shown that these mice have normal PPI (Paylor et al., 1998; Young et al., 2011). This discrepancy with drug studies may be due in part to methodological considerations, as another

study observed a deficit in auditory P50 gating in heterozygous α7-nAChR KO mice (Adams et al., 2008).

In terms of habituation, a study by Williams et al. (1975) has shown that habituation of reflexive behaviours, like startle, is mediated by separate mechanisms to those of habituation of motivated behaviours, such as exploratory behaviour and spontaneous locomotion. Pharmacological studies have suggested that acetylcholine (ACh) is very important for short- and long- term habituation of locomotion (Ikegami, 1994; Thiel et al., 1998; Giovannini et al., 2001). In particular, it has been shown that nicotinic receptors in the Nucleus Accumbens play an important role in early consolidation phases of long-term habituation of locomotion (Schildein et al., 2002). In contrast, reflexive behaviours historically have been suggested to be independent of ACh (Brown, 1976; Hughes, 1984). Indeed, there is no evidence to suggest that nicotinic receptors are involved in short-term habituation of startle (Brown, 1976; Hughes, 1984; Paylor et al., 1998). However, a recent study by Schmid et al. (2011) linked ACh to long-term habituation of startle, as mice with a general knock-down of the Vesicular Acetylcholine Transporter show clear long-term habituation deficits.

In this study, we therefore sought to evaluate the sensory filtering capacities of α7-nAChR KO mice using PPI, short- and long-term habituation of both startle and locomotion. We also treated animals with nicotine prior to testing in order to determine whether the enhancement of PPI is dependent on α7-nAChRs. Finally, we performed a spatial learning task in order to test whether sensorimotor gating deficits correlate with impairments in higher cognitive function. Recently, Singer et al. (2013) have demonstrated that in CB57BL/6 mice, PPI correlated with

working memory performance in the Morris water maze. We sought to reaffirm and expand on this correlation using the Barnes maze, which also emphasizes spatial learning and memory.

3.2 Methods

The order of testing was as follows: startle testing, locomotor box, Barnes maze and elevated plus maze for all animals.

3.2.1 Subjects

We used a commercially available mutant mouse line (B6.129S7Chrna7, stock no. 003232; Jackson Laboratories, Bar Harbour, ME, USA) that has a null mutation in the Chrna7tm1Bay gene, which encodes the α7-nAChR protein. The KO was produced by a deletion of the last three exons (8–10) of the *Chrna7* gene. The strain originated on a mixed129/SvEv and C57BL/6 background and has been backcrossed to the C57BL/6J line for at least eight (N8) generations. Control mice were age-matched wild-type (WT) C57BL/6J counterparts. Animals were cared for according to the ethical guidelines of the University of Western Ontario Animal Use Subcommittee and Canadian Council on Animal Care (CCAC). Mice were group housed, with a 12-h light–dark cycle with *ad libitum* food and water. Testing occurred at age 6–14 weeks during the light phase. For most tests, 18 male KO and WT mice (C57BL/6J from Jackson Laboratories) were used. Long-term habituation of startle and locomotor testing were tested with a separate batch of mice of both sexes (WT: n=21, 16 males/5 females; KO: n=14, 9 males/5 females).

3.2.2 Testing of the Acoustic Startle Response

All startle testing was completed using Med Associates sound-proofed startle boxes and associated software (Startle Reflex Version 5.95, St Albans, VT, USA). Figure 3.1 shows a

schematic representation of behavioural protocol. Animals were acclimated to the startle box for 5 min/day for 3 days with background noise (65 dB SPL, white noise). All testing sessions began with an acclimation phase (5 min, 65 dB SPL, white noise). On the final day of acclimation, animals also underwent an input/output (I/O) test to determine an appropriate gain setting for each individual animal (figure 3.2). An I/O function began with stimulation at 65 dB SPL (20-ms duration) and increased in 5 dB SPL steps to 120 dB SPL (for details see Schmid et al., 2011; Valsamis and Schmid, 2011). Once the gain was set it was kept constant throughout the remainder of the experiment.

For the next 5 days, the animals were tested once daily with the following behavioural protocol. Mice of each genotype (n=18/group, male) underwent PPI and short-term habituation testing. The protocol consisted of two blocks of trials (figure 3.1). Block I assessed habituation by presenting 33 trials of the startle pulse (20 ms white noise at 105 dB SPL and 15 s inter-trial interval, ITI). Block II assessed PPI. There were five different trial conditions (10/condition) for a total of 50 trials. All trials were presented in a pseudo-randomized order. The trial conditions were as follows: startle pulse alone trials (to determine baseline) and combinations of commonly accepted prepulses (75 or 85 dB SPL; 4 ms) at two different interstimulus intervals (ISIs; 30 or 100 ms). Separate animals were used for long-term habituation experiments (WT: n=21, 16 males/5 females; KO: n=14, 9 males/5 females). To examine long-term habituation, we employed the same acclimation schedule, but removed block II to prevent over presentation of startle stimuli, which can induce sensitization (Plappert et al., 1999).

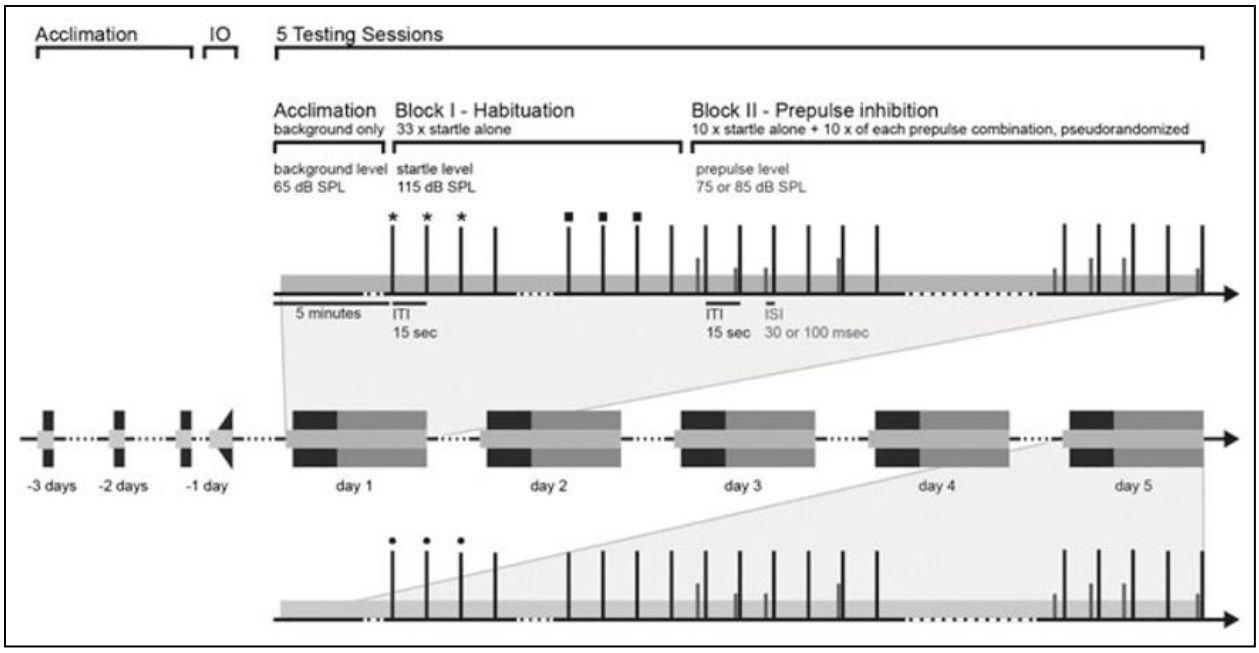

Figure 3.1 Testing of the Acoustic Startle Response

As shown in the graph, animals underwent 3 days of acclimation to startle boxes and background noise and an I/O function was measured on the third day. Next, they completed five subsequent testing days, where they were exposed to a 5-min acclimation period, a first block with 33 startle stimuli alone for measuring short-term habituation, and a second block with 50 trials, 10 trials each of startle stimuli alone, and any combination of 75 or 85 dB SPL prepulses, administered 30 or 100 ms before the startle pulse. Asterisks indicate the first three startle responses and squares indicate the last three responses in block I that were used to calculate the amount of short-term habituation in each animal. The dots indicate the first three startle responses on day 5 that were used along with the first three responses on day 1 (asterisks) to calculate the amount of long-term habituation. Please note that block II was omitted for testing long-term habituation as displayed in figure 3.5b,d, and a shortened program was used for testing the effects of nicotine injections (see section 3.2.2.1).

3.2.2.1 Nicotine Administration and Acoustic Startle Testing

To test the effect of nicotine on PPI, we used a shorter protocol to account for the drug's short half-life in mice blood plasma. Block I was reduced to 3 trials and block II to 30 trials (three different trial conditions, 10 trials per condition): startle pulse alone and 75 or 85 dB SPL prepulse (both with 30 ms ISI). Both WT (n=20, male) and KO (n=18, male) mice were given a single subcutaneous injection of either nicotine (1 mg/kg free base nicotine, nicotine hydrogen tartrate salt, Sigma Chemical Co., St. Louis, MO, USA, dissolved in phosphate-buffered saline, 0.9% NaCl) or saline immediately before behavioural testing. Each mouse was administered both treatments on separate days. We allotted a 2-day recovery period between treatments and the order of nicotine/saline administration was randomized and counterbalanced across genotypes.

3.2.2.2 Data and Statistical Analysis of Sensory Filtering and Sensorimotor Gating of the ASR

Startle magnitude was calculated as the maximal displacement of the movement-sensitive platform induced by the startle reflex following the startle pulse (arbitrary units). To detect differences in baseline startle between genotypes, we examined the I/O function, where all animals had the same gain factor, as well as initial startle values (average of the first three trials) on day 1 of testing for the group tested on long-term habituation. We used a two-way repeated measure analysis of variance (ANOVA) (genotype × sound level) for the I/O function and an unpaired Student's t-test for the latter group.

To analyse short-term habituation of startle, we calculated short-term habituation ratios (average of trials 28–30/average of first 3 trials; see figure 3.1, stimuli marked with squares and asterisks, respectively), and compared them using an unpaired Student's t test. To analyse long-term habituation, we normalized all data points to the average of the first three trials on day 1

for each individual mouse. We then used a three-way repeated measures ANOVA (day × genotype × sex) and post hoc unpaired Student's t -tests. We also calculated a long-term habituation ratio (average of first five trials on day 5/average of first five trials on day 1; corresponding to stimuli marked with asterisks and dots in figure 3.1), and compared ratios between genotypes using an unpaired Student's t-test.

The PPI was expressed as percent of prepulse inhibition (%PPI=[1−{startle magnitude with prepulse/baseline startle without prepulse}×100). We determined the average %PPI for each prepulse type and performed two-way ANOVA (trial type × genotype). We also calculated averages for each trial type per day and performed a two-way repeated measures ANOVA (genotype × day) in order to determine if PPI changed across days. When nicotine was administered, we performed a three-way repeated measures ANOVA (drug × genotype × prepulse) to determine changes in %PPI with drug treatment. Additionally, we subtracted PPI with nicotine administration from PPI with saline (%PPI nicotine−%PPI saline) for each animal, and used a one sample t-test to determine if the difference significantly differed from zero. To determine the effect of nicotine on baseline, we again subtracted baseline startle with saline treatment from baseline with nicotine and compared between genotypes with an unpaired t -test.

3.2.3 Locomotor Testing

We used locomotor behaviour to assess habituation of non-reflexive behaviours. To examine short-term habituation of locomotor behaviour mice (WT: n=6 males, KO: n=12 males) were placed in a locomotor box (Versamax, Columbia, OH, USA) to freely explore for 2h. Distance,

rearing, rest time and time spent in each quadrant of the box were measured. Data values were totalled and parsed into 5-min blocks, and a two-way repeated measures ANOVA (blocks of time × genotype) was performed to assess short-term habituation of locomotion.

Long-term habituation of locomotor behaviour was tested in separate mice (WT: n=15, 10 males/5 females; KO: n=13, 8 males/5 females), once daily for 15 min for 5 consecutive days. The values of the first 5-min block were analysed using a three-way repeated measures ANOVA (day × genotype × sex). Short-term habituation data were analysed in raw values, but for long-term habituation data was also normalized (activity/activity of first 5 min on day 1). This was for graphical representation and to reduce individual variability in locomotor behaviour as suggested by Thiel et al. (1998).

3.2.4 The Barnes Maze

The Barnes maze is designed to test spatial learning and memory in rodents. The protocol used for this test has been previously described by Sunyer et al. (2007). Mice (n=10/genotype, male) completed four trials per day on days 1–4 to ensure acquisition of the task. Trials were considered completed when a mouse entered the target hole, or when 3 min had passed. The inter-trial interval between testing was on average 20 min. On days 5 and 12 of testing, the animals completed probe trials to assess short- and long-term spatial memory, with the target hole covered to prevent entrance. Mice were given a single 90-second trial to freely explore the apparatus on probe days.

For all days, holes investigated by mice were tracked by ANY-Maze software (Version 4.82, Stoelting, Wood Dale, IL, USA). Investigation was defined as when a mouse hovered over a hole

with their nose (i.e. nose poke). The distance, latency to approach and enter target, as well as errors was tracked. In addition, on probe trial days, we measured the location of errors (based on distance to target). We defined two types of errors: a primary error was defined as any time the mouse investigated a hole that was not the target, and a secondary error was defined as the first instance a mouse investigated a non-target hole after investigating the target hole. Total errors were the sum of primary and secondary errors.

3.2.4.1 Data and Statistical Analysis of the Barnes Maze

Performance on days 1–4 showed how well the animals were able to learn the task. The values for each of the measures (distance, latency, or errors) examined were averaged over all four trials/day, in order to give us average performance for each day. Each measure was analysed using a two-way repeated measures ANOVA (day × genotype). For performance on day 1 only, a separate analysis was completed where latency to approach target was analysed by trial using a two-way repeated measures ANOVA (genotype × trial). This aimed to assess working memory performance, based on improvement across trials. Analysis of only day 1 was appropriate for this measure, to remove memory of the task as a potential confound. Performance on days 5 and 12 showed how well animals were able to recall the task in their short- or long-term spatial memory, respectively. To analyse this, separate two-way repeated measures ANOVAs were performed (hole × genotype).

3.2.5 Elevated Plus Maze

Both WT (n=14, male) and KO (n=14, male) mice were placed in the centre of the elevated plus maze. The apparatus contained four arms: two covered and two uncovered. Animals had 5

min to freely explore the maze. The number of entries, latency to enter and time spent in closed and open arms were digitally recorded by ANYMaze software (Version 4.82, Stoelting). Unpaired Student's t-tests were then used to determine differences between genotypes.

3.3 Results

3.3.1 α7-nACHR KO Mice Have Normal Startle Reactivity

Critical to our study was the ability of α7-nAChR KO mice to startle normally. We found that startle I/O functions did not differ between genotypes ($F_{(11,,403)}$=3.0, *p*=0.1; n=18/genotype: figure 3.2). Furthermore, baseline startle in block I (average of first three trials on day 1) and in block II (pulse-alone trials) did not differ between the genotypes (t_{34}=1.5, *p*=0.14; t_{34}=0.52, *p*=0.6, respectively).

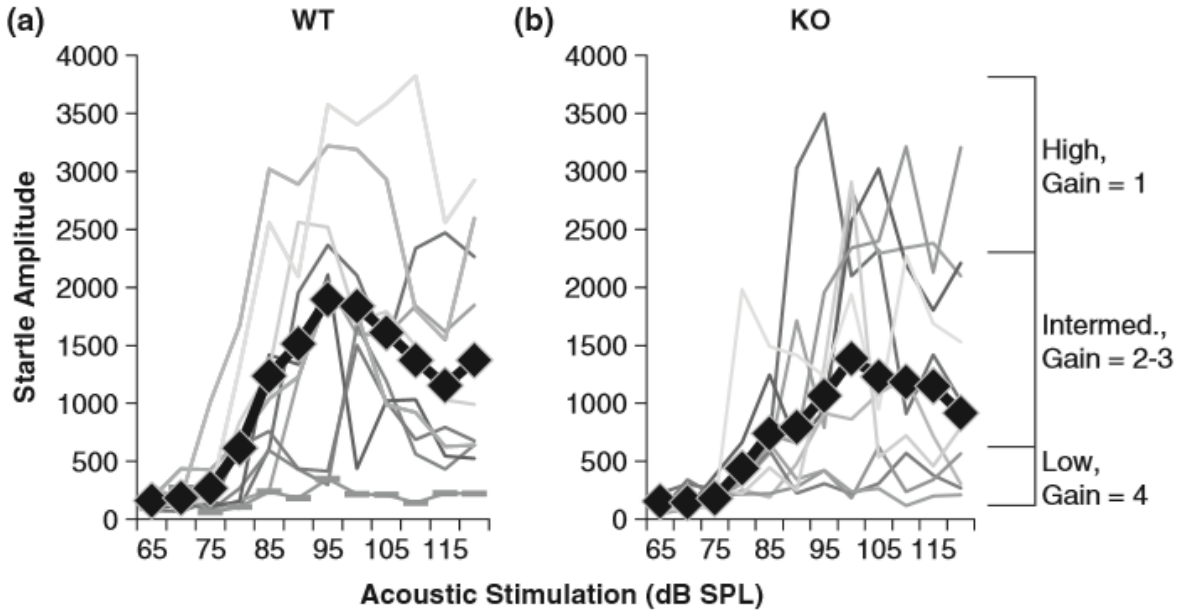

Figure 3.2 α7-nAChR KO Mice Have Normal Startle Reactivity

Representative I/O functions of startle response amplitudes for different startle stimulus intensities. Both genotypes display natural variability of startle magnitudes with very high and very low startling animals within each group. Based on startle amplitude (low, intermediate or high), a gain was prescribed as indicated in the figure (see also Methods). This allowed for accurate signal detection and prevented floor effects in low startling mice. The I/O function of (a) WT and (b) KO did not differ statistically. The solid black line indicates the average across all animals of the group (n=18/genotype, all male. Not all mice shown here for clarity).

3.3.2 Prepulse Inhibition is Mildly Impaired in α7-nAChR KO Mice

Eighteen male WT and KO animals underwent PPI testing. We observed a mild, but consistent impairment of PPI in α7-nAChR KO mice. In WT mice, when a 75 dB SPL prepulse preceded the startle pulse, startle was reduced by about 52.1% and 52.2% at the ISIs of 30 and 100 ms, respectively; whereas in KOs startle was only reduced by 38.5% and 37.2%, respectively. Therefore, PPI was significantly reduced in KO mice compared to WT ($F_{(1,34)}$=6.87, p=0.02, figure 3.3a) with a 75 dB SPL prepulse regardless of ISI. In all groups, PPI was stable across days of testing as there was no main effect of day at either ISI (30 ms ISI $F_{(4,175)}$=0.27, p=0.9; 100 ms $F_{(4,175)}$=1.0, p=0.4) or interaction between day and genotype (30 ms $F_{(4,700)}$=0.87, p=0.59; 100 ms $F_{(4,700)}$=0.27, p=0.9).

When a higher prepulse level of 85 dB SPL was used we did not observe any PPI differences between genotypes. In WT animals, startle was suppressed by 49% and 62.3% at 30 and 100 ms ISIs, respectively, and in KO mice by 44% and 53.8%, respectively. While there is still a trend of impaired PPI in KO, this failed to reach statistical significance ($F_{(1,34)}$=1.5, p=0.32, figure 3.3b). There was also no main effect of day (30 ms $F_{(4,175)}$=2.2, p=0.08; 100 ms $F_{(4,175)}$=0.48, p=0.8) or day by genotype interaction (30 ms $F_{(4,700)}$=1.9, p=0.11; 100 ms $F_{(4,700)}$=0.38, p>0.82).

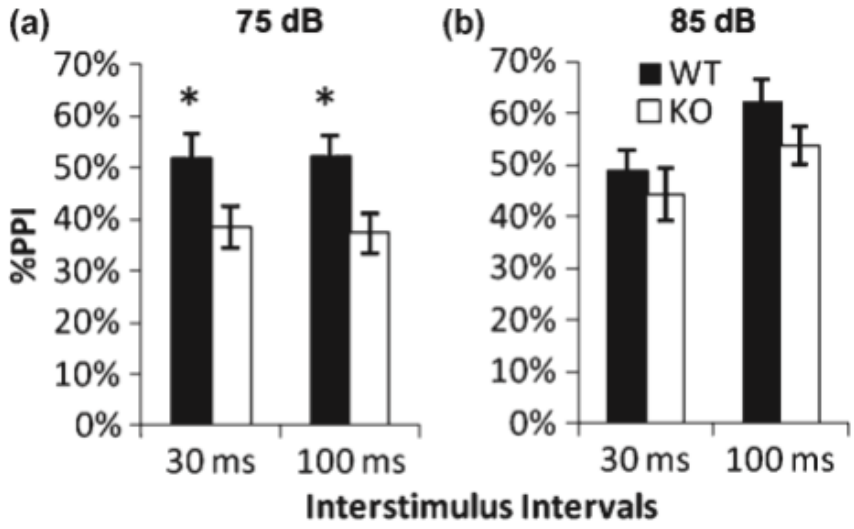

Figure 3.3 α7-nAChR KO Mice Have a Mild Impairment of PPI

(a) α7-nAChR KO mice were unable to suppress startle as effectively as WT across ISIs using a 75 dB SPL prepulse. (b) When an 85 dB SPL prepulse was used, α7-nAChR KO mice displayed the same ability as WT type to suppress startle across ISIs (n =18/genotype, male).

3.3.3 The α7-nAChR is Critical for Nicotine-Induced Enhancement of PPI

Many previous studies have shown that acute, systemic nicotine improved PPI; we wanted to investigate the role of the α7-nAChR in this enhancement. We injected WT (n =20, male) and α7-nAChR KO mice (n =18, male) with saline and nicotine (1 mg/kg, figure 3.4) before PPI testing with both prepulse intensities and an ISI of 30 ms. We performed a three-way ANOVA (drug × genotype × prepulse) and confirmed an impairment of PPI in KO mice at both prepulse intensities ($F_{(1,36)}$=5.5, p=0.025). The ANOVA did not detect a main effect of nicotine treatment ($F_{(1,36)}$=0.6, p=0.43) or interaction between genotype, drug, and prepulse ($F_{(1,36)}$=1.1, p=0.3). However, we did see that the drug tended to act differently according to the prepulse level, but

the drug by prepulse interaction just failed to reach significance ($F_{(1,36)}$=4.05, *p*=0.052). Generally, at the 75 dB SPL prepulse, we saw no effect of nicotine; PPI of WT mice was 45.2% with saline and 47% with nicotine administration. A similar trend was observed in the KO mice. When KO mice were administered saline, PPI was 34.6%, and when given nicotine it was 35.3% (figure 3.4a). At the higher prepulse of 85 dB SPL however, nicotine seemed to improve PPI in WT mice. When WT mice were administered saline, PPI was 43.6%, and when given nicotine it increased to 55.6%, whereas in the KO mice PPI was similar in both conditions: PPI with saline was 27.6% and with nicotine it was 30%. When we looked at the individually normalized changes in PPI, we found a significant improvement of PPI in WT (t_{17}=2.43, *p*=0.03), but not in KO animals (t_{17}=0.34, *p*=0.73, figure 3.4b). Two WT mice were eliminated from this analysis as outliers (±3 standard deviations from mean).

Nicotine also enhanced baseline startle amplitudes compared with saline treatment in WT animals (t_{17}=2.4, *p*=0.03), but not in KO mice (t_{17}=0.43, *p*=0.67). Once again, however, this effect was strongest when the data was analysed for individual changes in each mouse between saline and nicotine conditions. The WT mice showed an increased baseline startle when given nicotine, whereas KO mice showed no changes (mean change around 0, figure 4.4c). Changes in baseline startle were significantly different between genotypes (t_{34}=2.05, *p*=0.048).

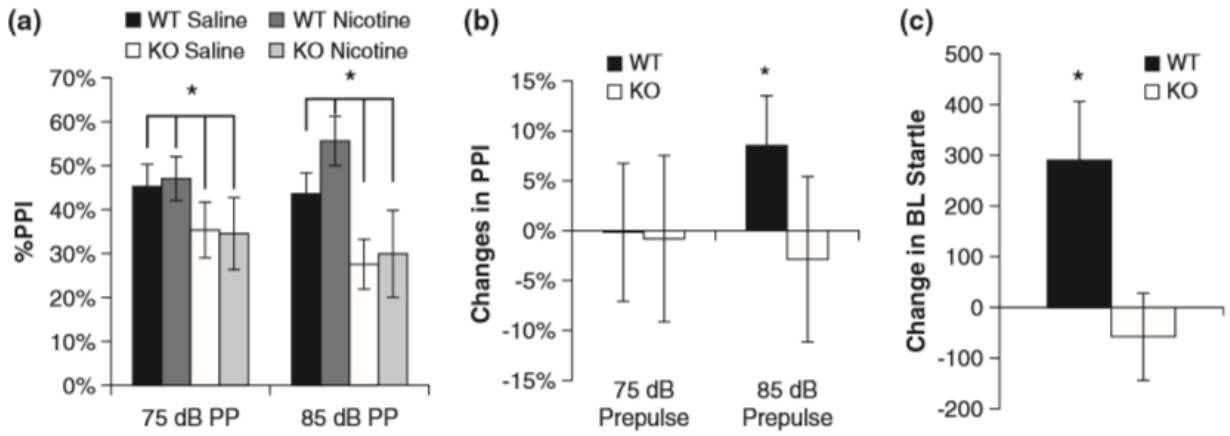

Figure 3.4 Nicotine-Induced Enhancement of PPI and Startle Magnitude is Absent in α7-nAChR KO Mice

(a) We reconfirmed that KO mice have impaired PPI compared with WT in both drug conditions and prepulses. Nicotine tends to enhance PPI at the 85 dB SPL prepulse in WT mice. We do not see enhancement at lower prepulse levels as PPI is generally weaker and more variable with a 75 dB SPL prepulse. (b) There was a significant effect of nicotine at 85 dB SPL prepulse on changes of PPI in WT, but not in KO mice. The asterisk denotes that the change in PPI (PPI nicotine−PPI saline) between drug conditions is significantly different from zero in WT but not KO mice. (c) The change in baseline (BL) startle amplitude between treatment conditions (nicotine baseline−saline baseline) is displayed for both genotypes. Nicotine enhanced baseline startle in WT animals, but not in α7-nAChR KO mice (n =18/genotype, male).

3.3.4 The Habituation of Reflexive and Non-Reflexive Behaviours is Unaltered in α7-nAChR KO Mice

We examined short- and long-term habituation of the startle response in male WT and α7-nAChR KO mice over 5 days of testing. Wild-type mice (n=18, male) show a progressive decrease in startle amplitude within a testing session, on average they reduced responding by about 27% by the end of block I (short-term habituation ratio=0.73). In KO mice (n=18, male) responses also decreased by about 23% (short-term habituation ratio=0.77).

The short-term habituation ratios between genotypes did not differ (t_{34}=1.7, p=0.1, figure 3.5a,b). We used a separate batch of animals to examine long-term habituation of startle with a shortened behavioural program in order to avoid overexposure. In WT mice (n=21, 16 males/5 females) average startle response decreased to 92%, and in KO mice (n=14, 9 males/5 females) to 91% within 5 days. These long-term habituation ratios did not significantly differ ($F_{(1,31)}$=1.6, p=0.22), neither was there any interaction between sex and genotype ($F_{(1,31)}$=0.6, p=0.46). When startle was normalized to day 1 of testing, the ANOVA revealed that startle significantly changed across days ($F_{(4,31)}$=21, p<0.001). There was no effect of sex ($F_{(1,31)}$=0.16, p=0.67) or genotype ($F_{(1,31)}$=0.01, p=0.79), or sex by genotype by day interaction ($F_{(4,28)}$=0.4, p=0.57; figure 3.5b).

Habituation of reflexive behaviours like the startle response is mediated by mechanisms distinct from habituation of non-reflexive, or motivated, behaviours like locomotion (that reflects exploratory behaviour). Therefore, we also examined short-term habituation of locomotion in WT (n=9, male) and α7-nAChR KO (n =12, male) mice. There was a significant decrease of distance travelled within the 2 h test session ($F_{(23,529)}$=9.9, p<0.001) with no effect of genotype ($F_{(1,23)}$=0.3, p=0.87) or interaction between genotype and time ($F_{(23,529)}$=1.26, p=0.2, figure 3.5c). Rearing

activity also significantly decreased within a test session ($F_{(23,506)}$=4.6, p<0.001), with no significant time by genotype interaction ($F_{(23,506)}$=1.0, p=0.49). For rearing analysis, one KO animal was eliminated as it never reared. Rest time tended to increase across time, but this failed to reach significance ($F_{(23,529)}$=1.3, p=0.14), with no interaction between time and genotype ($F_{(23,529)}$=1.2, p=0.22).

While the above data suggests that α7-nAChR KO mice have normal short-term habituation of locomotor behaviour, we did observe one difference between genotypes: KO mice spent significantly less time in the centre of the open field throughout testing compared with WT (t_{23}=1.82, p=0.04). They also tend to travel less in the centre, although this failed to reach significance (t_{23}=1.67, p=0.11).

To examine long-term habituation of locomotor behaviour, we used separate mice (WT: n=16, 9 males/5 females; KO: n=13, 8 males/5 females) and tested them in the locomotor box across 5days. We found that there was no difference in activity between genotypes ($F_{(1,25)}$=2.7, p=0.11) or sex ($F_{(1,25)}$=0.5, p=0.51). With normalized data (to day 1 of each animal) we found that the distance travelled significantly decreased across days ($F_{(4,100)}$=5.3, p=0.017), with no main effect of genotype ($F_{(1,25)}$=3.25, p>0.05), sex ($F_{(1,25}$)=0.8, p=0.37) or interaction of day, sex , and genotype ($F_{(4,100)}$=0.7, p=0.41). This shows that both genotypes had normal long-term habituation of locomotor behaviour, see figure 3.5d.

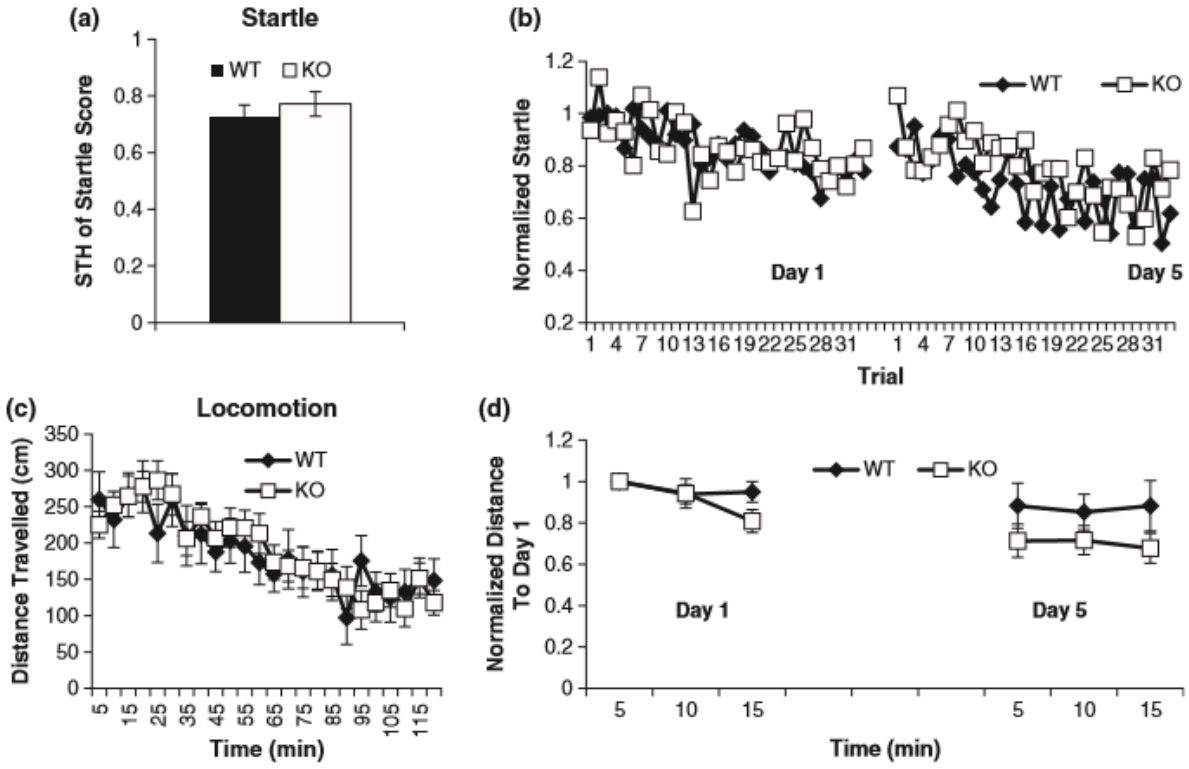

Figure 3.5 Habituation of Startle and Locomotion is Unaltered in α7-nAChR KO Mice

(a) Short-term habituation ratios for the startle response did not differ between genotypes. (b) Short- and long-term habituation of startle at days 1 and 5. In both genotypes startle amplitudes progressively decrease within a testing session and across testing sessions to a comparable degree. (c) Both genotypes show significant short-term habituation of locomotor behaviour, with activity greatly attenuated by the end of testing. (d) Both WT and KO mice decreased locomotor activity across days, displaying normal long-term habituation of locomotion (WT n=21, 16 males/5 females; KO n=14, 9 males/5 females). Overall, this suggests that the α7-nAChR was not critical for these behaviours.

3.3.5 α7-nAChR KO Mice Have No Impairments of Spatial Learning and Memory

To date, discrepancies exist whether α7-nAChR KO mice have normal or impaired spatial learning and memory, and spatial memory performance has been linked to PPI performance (Singer et al., 2013). Therefore, we tested male WT and α7-nAChR KO mice in the Barnes maze (n=10/genotype, male) in order to reassess spatial learning.

When we analysed the latency to approach target, we observed a main effect of day, which suggested that learning occurred across days ($F_{(5,114)}$=18.9, p<0.001). We found no main effect of genotype ($F_{(1,114)}$=0.1, p=0.78) or interaction between day and genotype ($F_{(5,570)}$=0.4, p=0.86), which indicates that both genotypes performed similarly (figure 3.6a). Furthermore, we observed normal activity levels as both genotypes travelled the same distance across days ($F_{(5,114)}$=2.6, p=0.14). While there was no difference in the number of primary errors made by genotypes ($F_{(5,114)}$=4.4, p=0.07), KO mice tended to make significantly less total and secondary errors ($F_{(5,114)}$=5.1, p=0.036; $F_{(5,114)}$=17.6, p=0.015, respectively). Consequently, they also took significantly less time to enter the target (as opposed to approach it) than WT mice ($F_{(3,76)}$=10.2, p=0.005), despite both genotypes improvement across days of testing ($F_{(3,76)}$=5.1, p=0.003). Additionally, as Singer et al. (2013) found the strongest correlation between PPI and spatial working memory; we also examined improvements on day 1 across trials. Both genotypes considerably improved across trials ($F_{(3,54)}$=6.53, p=0.001) with no difference between genotypes ($F_{(1,18)}$=0.09, p=0.78, figure 3.6b).

We found a significant preference for the target hole on days 5 ($F_{(19,380)}$=39, p<0.001) and 12 ($F_{(19,380)}$=23.3, p<0.001) with no main effect of genotype ($F_{(1,18)}$=0.01, p=0.93; $F_{(1,18)}$=1.1, p=0.32, days 5 and 12, respectively, figure 3.6c,d). Furthermore, latency to approach the target

did not differ between genotypes on days 5 or 12 as there was no effect of day ($F_{(1,18)}$=0.18, p=0.68),genotype ($F_{(1,18)}$=0.12, p=0.73) or interaction between day and genotype ($F_{(1,18)}$=0.14, p=0.71), which shows that α7-nAChR KO mice have normal retention of spatial tasks.

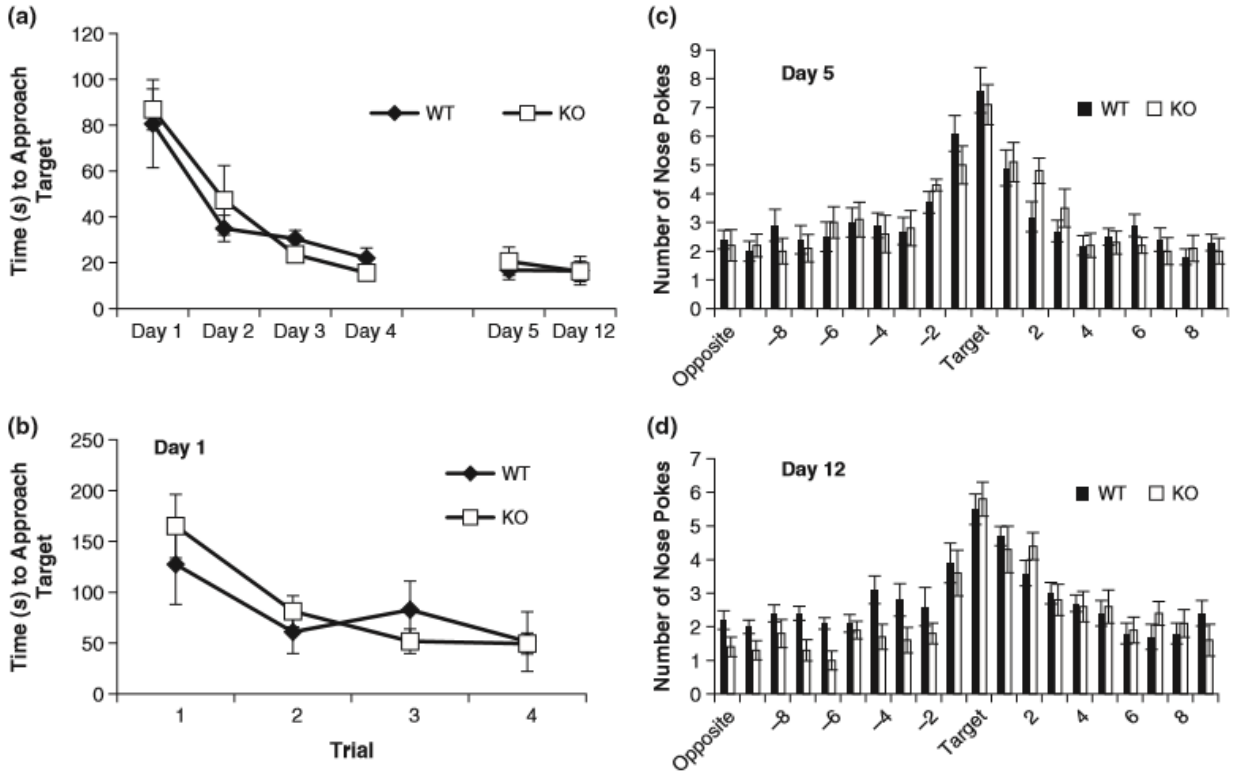

Figure 3.6 α7-nAChR KO Mice Have Normal Spatial Learning and Memory

(a) α7-nAChR mice show normal acquisition of a spatial task, as both genotypes significantly improved performance on training days 1–4. Unchanged performance on days 5 and 12 suggests that both genotypes accurately remembered the task. (b) The analysis of spatial working memory during the first training sessions within day 1. Both genotypes show similar times to approach the target on day 1. (c) Number of nose pokes on the different holes on the maze. Both WT and α7-nAChR KO mice show a preference for the target holes on day 5 and (d) day 12, suggesting that α7-nAChR KO mice have normal short- and long-term spatial memory (n=10/genotype, male).

3.3.6 Elevated Plus Maze Testing in α7-nAChR KO Mice

The fact that α7-nAChR KO mice spent less time in the centre of the locomotor box and seem to have an increased drive to enter the target drop box in the Barnes maze may be indicative of an increased level of anxiety in these mice. Therefore, we decided to directly assess anxiety using the elevated plus maze (n=14/genotype, all male). We found that total distance travelled (WT=17.0 ± 0.8 m, KO=16.5 ± 1 .0 m; t_{26}=0.3, *p*=0.76), latency to enter the open arm (WT=4.5 ± 1.9 s, KO=4.9 ± 2.6 s; t_{24}=0.3, *p*=0.87) and number of entries into closed (WT=28.0 ± 1.3, KO=27.4 ± 2.1; t_{26}=0.25, *p*=0.80) or open arms (WT=16.1 ± 1.2, KO=15.4 ± 1.1; t_{26}=0.5, *p*=0.63) did not differ between genotypes. However, we did find that KO animals spent more time in closed vs. open arms compared with WT (WT=152 ± 14.9 s, KO=199.1 ± 8.0 s; t_{24}=2.8, *p*=0.001), which suggests that they are more anxious than their WT littermates.

3.4 Discussion

The aim of this study was to understand the role of α7nAChR in sensory filtering and sensorimotor gating mechanisms and how they relate to higher cognition.

3.4.1 Prepulse Inhibition

We observed that α7-nAChR KO mice had a mild, but consistent and significant impairment of PPI. The KO mice consistently show reduced PPI at the 75 dB SPL prepulse, regardless of ISI. At the higher prepulse level of 85 dB SPL, PPI differences failed to reach significance in one of two groups. Generally, PPI is more robust at higher prepulse levels, and so we suggest that this impairment is mild and therefore most apparent when PPI is not at its

maximum. As pharmacological data suggest that α7-nAChR plays a very important role in PPI, our observation of a mild deficit may in part be due to compensation by other nicotinic receptors in our KO model. Supportive of this idea, Adams et al. (2008) observed P50 auditory gating deficits only in heterozygous KO mice.

Our observed PPI deficit does not match with the results of previous α7-nAChR KO mice studies. Both Paylor et al. (1998) and Young et al. (2011) observed normal PPI in KO mice. Both studies also used a α7-nAChR subunit null mutation, generated in a mixed 129/SvEv C57BL/6J line that were backcrossed onto the C57BL/6J strain for at least six generations. As our line (purchased from Jackson Laboratories) matches this background, genetic differences are unlikely to account for our observed results. The explanation for the discrepancy might lie in the differences between experimental protocols. Prepulse levels and ISIs were the same in all studies; however, both Paylor et al. (1998) and Young et al. (2011) used male and female mice, and found a main effect of sex on PPI and baseline startle. This might have increased the variability of their data and thereby occluded a mild PPI deficit. Reduced variability by only using male mice makes our experiment more apt to detect this deficit in PPI.

Additionally, in past studies, animals may not have been sufficiently habituated to the startle stimulus prior to PPI testing. Without sufficient prior exposure to startle stimuli alone, short-term habituation interferes with PPI measurements, especially with the first trials, thereby further increasing the variability. Finally, Young et al. (2011) did not normalize PPI measurements for each mouse, which greatly increases the inherent variability between mice (see figure 3.2). In fact, they show a higher average baseline startle magnitude in KO mice compared with WT, but the same startle response magnitude when a prepulse is present, which may have reflected a

disruption of PPI in KO mice had normalized PPI been calculated for each animal. Additionally, studies have shown that differences in baseline startle also influence PPI, particularly when data is not normalized (Csomor et al., 2008).

3.4.2 Nicotine-Induced Enhancement of PPI

Apart from the fact that α7-nAChRs play a minor role in PPI, we also observed that they are critical for nicotine-induced enhancement of startle amplitude and PPI. Although the overall ANOVA failed to yield a significant effect of nicotine, we did observe a slight, but significant enhancement of PPI with nicotine when %PPI was normalized to reflect changes from the saline condition (%PPI nicotine−%PPI saline), which is in accordance with the previous studies (Gould et al., 2005). The ANOVA likely failed to reach significance because of the high number of factors involved in the analysis and because of a ceiling effect, as WT mice were already performing well with saline administration. By reducing variability, via normalizing to the saline condition, this effect was strengthened and able to achieve significance.

Where this nicotine effect is mediated is not fully understood yet. Nicotine may simply amplify the contribution of the α7-nAChR to PPI, thereby causing PPI enhancement. Startle-mediating neurons of the Caudal Pontine Reticular Nucleus (PnC) receive cholinergic input from the midbrain that is assumed to mediate PPI (Fendt and Koch, 1999; Bosch and Schmid, 2006, 2008). Potentially, α7-nAChRs in the PnC could directly modulate baseline startle effects and possibly even PPI. Alternatively, many PPI-modulating brain areas are known to express α7-nAChRs, including the Prefrontal Cortex, Hippocampus, Ventral Tegmental Area and Nucleus Accumbens (Gotti et al., 1997; Paterson and Nordberg, 2000). Future studies should seek to

understand where this effect is occurring through localized injections of α7-nAChR agonists and antagonists.

Studies estimate that smoking rates in Schizophrenic populations are two to four times greater when compared with the normal population (Hughes et al., 1986; Leonard et al., 2000). In both healthy and Schizophrenic patients, PPI improved after smoking (Kumari et al., 1997; Kumari et al., 2001), which may indicate that Schizophrenics are smoking as a form of self-medication (Kumari and Postma, 2005). Our study indicates that the α7-nAChRs are at least partially mediating aspects of the initial beneficial effects of nicotine. It is important to note, however, that we only provide evidence that α7-nAChRs are critical for acute effects of nicotine. Chronic nicotine is known to alter nicotinic responses and receptor levels; therefore, the situation may be different in smokers.

3.4.3 Habituation of Reflexive and Non-Reflexive Behaviours

In accordance with previous literature, we did not find that the α7-nAChR was involved in short- or long-term habituation of the startle response. A recent study by Schmid et al. (2011) showed that the neurotransmitter acetylcholine was involved in long-term habituation of startle, but we did not find any influence of the α7-nAChR on long-term habituation, which suggests that the effect is mediated by other cholinergic receptors.

Furthermore, we did not find any evidence that the α7-nAChR is involved in short- or long-term habituation of locomotor behaviour. Previous studies suggested that nAChRs were important for the consolidation of long-term habituation of locomotion (Schildein et al., 2002).

Again, our study indicates that a different nicotinic receptor subtype might be responsible for the previously reported effects.

Overall, we found that the α7-nAChRs are not necessary for habituation of reflexive or non-reflexive behaviours, although, as with all constitutive KO mice, compensation by the knockout model cannot be ruled out.

3.4.4 Spatial Learning and Higher Cognition

We tested whether deficits in sensory filtering and sensorimotor gating correlate with deficits in higher cognitive processes, especially in spatial working memory tasks, as previously shown (Erwin et al., 1998; Singer et al., 2013). Studies of spatial learning and memory in the α7-nAChR KO mouse have been inconclusive in the past (Paylor et al., 1998; Curzon et al., 2006; Fernandes et al., 2006). In accordance with the findings of Paylor et al. (1998), we found normal spatial learning and memory in α7-nAChR KO mice. This was rather surprising as the α7-nAChR is known to be highly expressed in the Hippocampus (Freedman et al., 1995; Guan et al., 1999). Again, there may be compensation by other nicotinic receptors in our KO model as Curzon et al. (2006) found that a deficit in spatial learning existed in an inducible KO model. However, a recent study by Winterer et al. (2013) also failed to show improvements in P50 sensory gating in schizophrenic patients using an α7-nAChR positive allosteric modulator, which implied that the role of α7-nAChR in higher cognition is still unclear.

We found mildly impaired PPI in α7-nAChR KO mice but normal spatial learning and memory, and so did not observe the hypothesized correlation between sensorimotor gating deficits and higher cognitive function. The difference between our and Singer et al.'s (2013)

findings could be due to task differences as their protocol emphasized spatial working memory. We assessed improvement of performance on day 1 to examine working memory, but found no differences between genotypes. Conversely, recent data suggests that impaired attention is central in the cognitive deficits observed in α7-nAChR KO mice (Young et al., 2007); therefore, the observed PPI deficit might correlate better with disruptions in attentional-based tasks.

3.4.5 Anxiety

Interestingly, we found that KO mice were significantly faster to enter the target during Barnes maze testing, despite no genotype differences in latency to approach target. Accordingly, we also observed that KOs were significantly less likely to make secondary errors. During locomotor testing KO mice spent less time in the centre of the locomotor box. Accumulating this evidence, we suggest that an increased level of anxiety in α7-nAChR KO mice may explain these findings. Increased anxiety would make the mice more motivated to enter the target in the Barnes maze instead of exploring other holes, as well as stay closer to the walls in the locomotor box. In the elevated plus maze test, however, most parameters were similar between genotypes, except the time spent in closed vs. open arms. This finding suggests that α7-nAChR KO mice may be slightly more anxious. It should be noted that we also ran light/dark box testing on a separate batch of animals (data not shown), but increased anxiety could not be reconfirmed.

Other studies have noted no changes in anxiety compared to wildtype mice (Paylor et al., 1998; Fernandes et al., 2006); however, one study observed that α7-nAChR mice had longer freezing times during conditioning tasks, which correlates with heightened anxiety (Davis and Gould, 2007). Additionally, a recent study by Pandya and Yakel (2013) found that in rats, high

doses of an α7-nAChR agonist (10mg/kg, PNU-282987) had anxiogenic effects during open field testing that could be rescued by serotonin (5-HT1a) antagonism. Clearly, the role of α7-nAChR in anxiety needs further elucidation.

3.4.6 Conclusions

In summary, we have shown that α7-nAChRs play a (small) role in PPI, and are critical to nicotine-induced enhancement of both PPI and startle magnitude. We did not find any evidence to suggest that this receptor is involved in habituation of reflexive or non-reflexive behaviours. We also found that α7-nAChR KO mice had normal spatial learning and memory, consistent with most previous studies, and that they may have had slightly heightened anxiety. Future studies will seek to understand the mechanisms underlying the α7-nAChR effects on startle and PPI.

3.5 References

Acri JB (1994) Nicotine modulates effects of stress on acoustic startle reflexes in rats: dependence on dose, stressor and initial reactivity. Psychopharmacology (Berl) 116:255-265.

Acri JB, Morse DE, Popke EJ, Grunberg NE (1994) Nicotine increases sensory gating measured as inhibition of the acoustic startle reflex in rats. Psychopharmacology (Berl) 114:369-374.

Adams CE, Yonchek JC, Zheng L, Collins AC, Stevens KE (2008) Altered hippocampal circuit function in C3H alpha7 null mutant heterozygous mice. Brain Res 1194:138-145.

Bosch D, Schmid S (2006) Activation of muscarinic cholinergic receptors inhibits giant neurones in the caudal pontine reticular nucleus. Eur J Neurosci 24:1967-1975.

Bosch D, Schmid S (2008) Cholinergic mechanism underlying prepulse inhibition of the startle response in rats. Neuroscience 155:326-335.

Brown CP (1976) Two types of habituation in chicks: differential dependence on cholinergic activity. Pharmacol Biochem Behav 4:235-238.

Csomor PA, Yee BK, Vollenweider FX, Feldon J, Nicolet T, Quednow BB (2008) On the influence of baseline startle reactivity on the indexation of prepulse inhibition. Behav Neurosci 122:885-900.

Curzon P, Anderson DJ, Nikkel AL, Fox GB, Gopalakrishnan M, Decker MW, Bitner RS (2006) Antisense knockdown of the rat alpha7 nicotinic acetylcholine receptor produces spatial memory impairment. Neurosci Lett 410:15-19.

Davis JA, Gould TJ (2007) beta 2 subunit-containing nicotinic receptors mediate the enhancing effect of nicotine on trace cued fear conditioning in C57BL/6 mice. Psychopharmacology 190:343-352.

Dunlop J, Lock T, Jow B, Sitzia F, Grauer S, Jow F, Kramer A, Bowlby MR, Randall A, Kowal D, Gilbert A, Comery TA, Larocque J, Soloveva V, Brown J, Roncarati R (2009) Old and new pharmacology: positive allosteric modulation of the alpha7 nicotinic acetylcholine receptor by the 5-hydroxytryptamine(2B/C) receptor antagonist SB-206553 (3,5-dihydro-5-methyl-N-3-pyridinylbenzo[1,2-b:4,5-b']di pyrrole-1(2H)-carboxamide). J Pharmacol Exp Ther 328:766-776.

Erwin RJ, Turetsky BI, Moberg P, Gur RC, Gur RE (1998) P50 abnormalities in schizophrenia: relationship to clinical and neuropsychological indices of attention. Schizophr Res 33:157-167.

Faraday MM, O'Donoghue VA, Grunberg NE (1999) Effects of nicotine and stress on startle amplitude and sensory gating depend on rat strain and sex. Pharmacol Biochem Behav 62:273-284.

Fendt M, Koch M (1999) Cholinergic modulation of the acoustic startle response in the caudal pontine reticular nucleus of the rat. Eur J Pharmacol 370:101-107.

Fernandes C, Hoyle E, Dempster E, Schalkwyk LC, Collier DA (2006) Performance deficit of alpha7 nicotinic receptor knockout mice in a delayed matching-to-place task suggests a mild impairment of working/episodic-like memory. Genes Brain Behav 5:433-440.

Freedman R, Hall M, Adler LE, Leonard S (1995) Evidence in postmortem brain tissue for decreased numbers of hippocampal nicotinic receptors in schizophrenia. Biol Psychiatry 38:22-33.

Giovannini MG, Rakovska A, Benton RS, Pazzagli M, Bianchi L, Pepeu G (2001) Effects of novelty and habituation on acetylcholine, GABA, and glutamate release from the frontal cortex and hippocampus of freely moving rats. Neuroscience 106:43-53.

Gotti C, Fornasari D, Clementi F (1997) Human neuronal nicotinic receptors. Prog Neurobiol 53:199-237.

Gould TJ, Rukstalis M, Lewis MC (2005) Atomoxetine and nicotine enhance prepulse inhibition of acoustic startle in C57BL/6 mice. Neurosci Lett 377:85-90.

Guan ZZ, Zhang X, Blennow K, Nordberg A (1999) Decreased protein level of nicotinic receptor alpha7 subunit in the frontal cortex from schizophrenic brain. Neuroreport 10:1779-1782.

Hughes JR, Hatsukami DK, Mitchell JE, Dahlgren LA (1986) Prevalence of smoking among psychiatric outpatients. Am J Psychiatry 143:993-997.

Hughes RN (1984) Cholinergic and monoaminergic substrates of startle habituation. Life Sci 34:2101-2105.

Ikegami S (1994) Behavioral impairment in radial-arm maze learning and acetylcholine content of the hippocampus and cerebral cortex in aged mice. Behav Brain Res 65:103-111.

Ingram N, Martin S, Wang JH, van der Laan S, Loiacono R, van den Buuse M (2005) Interaction of corticosterone and nicotine in regulation of prepulse inhibition in mice. Neuropharmacology 48:80-92.

Koch M, Klarner A, Schnitzler HU (1999) Lesions of the rat piriform cortex prevent long-lasting sensorimotor gating deficits induced by stimulation of the ventral hippocampus. Neurosci Lett 275:195-198.

Kumari V, Postma P (2005) Nicotine use in schizophrenia: the self medication hypotheses. Neurosci Biobehav Rev 29:1021-1034.

Kumari V, Soni W, Sharma T (2001) Influence of cigarette smoking on prepulse inhibition of the acoustic startle response in schizophrenia. Hum Psychopharmacol 16:321-326.

Kumari V, Cotter PA, Checkley SA, Gray JA (1997) Effect of acute subcutaneous nicotine on prepulse inhibition of the acoustic startle reflex in healthy male non-smokers. Psychopharmacology (Berl) 132:389-395.

Leonard S, Breese C, Adams C, Benhammou K, Gault J, Stevens K, Lee M, Adler L, Olincy A, Ross R, Freedman R (2000) Smoking and schizophrenia: abnormal nicotinic receptor expression. Eur J Pharmacol 393:237-242.

Pandya AA, Yakel JL (2013) Activation of the alpha7 nicotinic ACh receptor induces anxiogenic effects in rats which is blocked by a 5-HT(1)a receptor antagonist. Neuropharmacology 70:35-42.

Paterson D, Nordberg A (2000) Neuronal nicotinic receptors in the human brain. Prog Neurobiol 61:75-111.

Paylor R, Nguyen M, Crawley JN, Patrick J, Beaudet A, Orr-Urtreger A (1998) Alpha7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensorimotor gating: a behavioral characterization of Acra7-deficient mice. Learn Mem 5:302-316.

Schildein S, Huston JP, Schwarting RK (2002) Open field habituation learning is improved by nicotine and attenuated by mecamylamine administered posttrial into the nucleus accumbens. Neurobiol Learn Mem 77:277-290.

Schmid S, Azzopardi E, De Jaeger X, Prado MA, Prado VF (2011) VAChT knock-down mice show normal prepulse inhibition but disrupted long-term habituation. Genes Brain Behav 10:457-464.

Simosky JK, Stevens KE, Kem WR, Freedman R (2001) Intragastric DMXB-A, an alpha7 nicotinic agonist, improves deficient sensory inhibition in DBA/2 mice. Biol Psychiatry 50:493-500.

Singer P, Hauser J, Llano Lopez L, Peleg-Raibstein D, Feldon J, Gargiulo PA, Yee BK (2013) Prepulse inhibition predicts working memory performance whilst startle habituation predicts spatial reference memory retention in C57BL/6 mice. Behav Brain Res 242:166-177.

Thiel CM, Huston JP, Schwarting RK (1998) Hippocampal acetylcholine and habituation learning. Neuroscience 85:1253-1262.

Valsamis B, Schmid S (2011) Habituation and prepulse inhibition of acoustic startle in rodents. J Vis Exp:e3446.

Wallace TL, Callahan PM, Tehim A, Bertrand D, Tombaugh G, Wang S, Xie W, Rowe WB, Ong V, Graham E, Terry AV, Jr., Rodefer JS, Herbert B, Murray M, Porter R, Santarelli L, Lowe DA (2011) RG3487, a novel nicotinic alpha7 receptor partial agonist, improves cognition and sensorimotor gating in rodents. J Pharmacol Exp Ther 336:242-253.

Williams JM, Hamilton LW, Carlton PL (1975) Ontogenetic dissociation of two classes of habituation. J Comp Physiol Psychol 89:733-737.

Winterer G, Gallinat J, Brinkmeyer J, Musso F, Kornhuber J, Thuerauf N, Rujescu D, Favis R, Sun Y, Franc MA, Ouwerkerk-Mahadevan S, Janssens L, Timmers M, Streffer JR (2013) Allosteric alpha-7 nicotinic receptor modulation and P50 sensory gating in schizophrenia: a proof-of-mechanism study. Neuropharmacology 64:197-204.

Young JW, Minassian A, Paulus MP, Geyer MA, Perry W (2007) A reverse-translational approach to bipolar disorder: rodent and human studies in the Behavioral Pattern Monitor. Neurosci Biobehav Rev 31:882-896.

Young JW, Meves JM, Tarantino IS, Caldwell S, Geyer MA (2011) Delayed procedural learning in alpha7-nicotinic acetylcholine receptor knockout mice. Genes Brain Behav 10:720-733.

3.6 Link between Chapter 3 and 4

Our last study suggested that the α7-nAChR has a minor role in the mediation of PPI, but no role in the LTH of the ASR. Our first study (Chapter 2) also uncovered no major deficits in PPI in mice with deficient cholinergic tone. Together, our evidence seems to suggest that perhaps ACh is not a major contributor to PPI, in contrast to what has been traditionally assumed. However, as both our models used congenital and chronic manipulations of cholinergic activity, it is hard to rule out what role compensatory mechanisms may have played.

Historically the PPT has been hypothesized to provide the cholinergic input necessary for PPI. The PPT is a heterogeneous structure, composed of glutamatergic, GABAergic and cholinergic neurons. Deficits in PPI have been reliably induced after non cell-type specific lesions of the PPT, but this deficit was attributed to the loss of cholinergic cells. As our previous chapters have only seen a mild link between PPI and ACh, we wanted to re-evaluate the role of the cholinergic neurons in the PPT in PPI. To do so, we used optogenetics and DREADDs, which both specifically targeted only cholinergic neurons of the PPT. Furthermore, we were able to transiently manipulate the activity of cholinergic PPT neurons, reducing the impact of compensation.

4. Chapter 4

Cholinergic Midbrain Neurons Modulate Startle Magnitude, but Not Prepulse Inhibition

4.1 Introduction

We are constantly bombarded with incoming sensory information from our five senses that our brain needs to process. How we process sensory information is critical for our daily functioning. Appropriate reduction of sensory information reaching conscious awareness and removal of behavioural responding to unnecessary sensory information is therefore highly adaptive. An inability to filter inundating sensory information can overwhelm our cognitive capabilities. Deficits in sensory filtering and sensorimotor gating occurs in many mental illnesses, including schizophrenia, obsessive compulsive disorder, and autism spectrum disorders (Braff and Geyer, 1990; Ornitz et al., 1993; Wynn et al., 2004; Hoenig et al., 2005). Examples of sensory filtering processes include habituation and prepulse inhibition (PPI), both can be studied in humans and pre-clinical animal models using the acoustic startle response (ASR).

Habituation is defined as the progressive decrease in startle magnitude after repeated exposure to the startle-inducing sound. This reduces the cognitive and motor burden of repetitive sensory information (Koch, 1999). Habituation can occur within a day (short-term habituation) or across days (long-term habituation). Sensitization is the opposite of habituation; it reflects the increase in behavioural responding after repeated exposure and is mediated through an independent mechanism (Groves and Thompson, 1970). In any case, it is important to keep in mind that an individual's behavioural output is always the sum of these influences; therefore, they cannot be studied independently.

PPI occurs when the presentation of a sensory stimulus (prepulse) prior to a startle-inducing sound reduces the magnitude of the startle response compared to when that same startling sound is presented alone. This is termed sensorimotor gating and it is thought to protect

ongoing neuronal processing from distractive interference by the secondary stimulus (Koch, 1999). Alternatively, the prepulse is predicted to coordinate approach behaviours by facilitating orienting responses such as eye-saccades through activation of neurons in the superior colliculus while simultaneously inhibiting startle-mediating neurons in the brainstem. In this way, PPI can thought of as a very early form of response selection (Yeomans, 2012).

The timing between the prepulse and startle pulse, termed the interstimulus interval (ISI) can greatly impact the amount PPI (Yeomans et al., 2010). The behavioural opposite of PPI is paired pulse facilitation (PPF), where the presentation of a pulse prior to a startling sound enhances startle magnitude. PPI generally occurs at ISIs of 20-1000 ms, whereas PPF occurs at short (>10 ms) or very long ISIs of more than 1 second (Ison et al., 1973; Graham, 1975; Ison et al., 1997). There is evidence to suggest that PPF opposes PPI by an independent mechanism to determine behavioural output in a manner similar to habituation and sensitization (Ison et al., 1973). However this may only be the case at ISIs that border the induction of PPI or PPF (around 10 ms).

PPI and habituation both lead to an attentuation of sensory signals, but they are thought to be mediated by very different brain mechanisms. Also, habituation of reflexive compared to non-reflexive behaviours has been observed to be differentially regulated (Williams et al., 1975; Hughes, 1984). Prepulse inhibition of the ASR has been very reliably shown to be impacted by manipulations of ACh (Fendt and Koch, 1999; Hohnadel et al., 2007; Yeomans et al., 2010; Pinnock et al., 2015); whereas cholinergic manipulations have been shown to differentially regulate habituation of reflexive (ASR) versus non-reflexive (locomotor) behaviours (Hughes, 1984; Thiel et al., 1998; Schildein et al., 2002). While there appears to be no role of ACh in the

short-term habituation of the ASR (Hughes, 1984), recent evidence suggested it may be important for long-term habituation of the ASR (Schmid et al., 2011). Regarding non-reflexive habituation, ACh has been linked to both long- (Thiel et al., 1998) and short-term habituation (Lamprea et al., 2003). Understanding the source of this cholinergic modulation as well as the receptors involved may help uncover more about this differential regulation on habituation.

It has been a long-standing hypothesis within the field that the pedunculopontine tegmental nucleus (PPT) provides cholinergic inhibition onto startle-mediating neurons of the brainstem, more specifically the giant neurons of the caudal pontine reticular formation (PnC) (Koch et al., 1993; Swerdlow and Geyer, 1993; Fendt and Koch, 1999; Bosch and Schmid, 2006, 2008). This cholinergic inhibition is the hypothesized mechanism underlying PPI; there has been no suggestion that it has any role in the habituation of the ASR or locomotion, but this has been largely untested.

Recent evidence has challenged the cholinergic midbrain hypothesis of PPI. For example, a conditional knock-out of cholinergic function in the midbrain (including the PPT, laterodorsal tegmental (LDT) and parabigeminal nucleus (PGB)) caused mice to have *improved* PPI (Machold, 2013). Additionally, a study by MacLaren et al. (2014) was the first to selectively lesion the cholinergic cells of the PPT and examine sensorimotor gating. Specific lesions of cholinergic PPT neurons profoundly reduced startle magnitude, but left PPI intact. Similar to previous studies which originally provided the foundation for this hypothesis (Koch et al., 1993; Swerdlow and Geyer, 1993), they found that non-specific lesions to the PPT still reliably induced PPI deficits. Their observed reduction in startle magnitude was counter-intuitive to the hypothesized

inhibitory role of cholinergic neurons and their mediation of PPI, however earlier studies also suggested that these neurons may modulate startle magnitude.

Stimulation of the ventral amygdalofugal pathway and ventral midbrain at sites that included (but were not limited to) the PPT have been shown to enhance, or even induce, startle-like responses (Yeomans and Pollard, 1993). Furthermore, electrical stimulation of PPT neurons caused prolonged excitation of PnC neurons in the cat (Garcia-Rill et al., 2001; Homma et al., 2002), and substance P-induced excitation of giant neurons was augmented by cholinergic agonism (Kungel et al., 1994). This is particularly interesting as substance P has been strongly linked to sensitization of startle (Krase et al., 1994), and is known to be co-expressed by a subset of cholinergic neurons in the PPT which directly innervated the PnC (Kungel et al., 1994).

Together, these studies suggest that the cholinergic midbrain hypothesis of PPI and cholinergic inhibition of startle in general, may need to be re-examined. With the advent of new technologies, namely chemogenetic and optogenetic approaches, we aimed to revisit the impact of PPT cholinergic projections to the startle pathway with improved cell-type and temporal resolution that was previously unachievable. We specifically wanted to test the impact of cholinergic PPT activity on PPI and habituation of both the ASR and locomotion.

4.2 Methods

This chapter is composed of 3 parts with overlapping but distinct methodology. Firstly, we verified the phenotype of our transgenic rat line (discussed below) to ensure it was an appropriate model for sensory filtering and sensorimotor gating testing. We then used this line to inhibit (section 4.2.3) or activate (section 4.2.4) cholinergic PPT neurons during behavioural

tasks. For each experimental approach we completed ASR testing, as well as a positive behavioural control task, conditioned place preference. In cholinergic inhibition studies, we also examined the habituation of locomotor behaviour. Finally, we completed immunohistochemistry and/or electrophysiology as proof of functional neuronal inhibition or activation.

4.2.1 Subjects

For this study we used a hemizygous transgenic rat line (Long Evans-Tg(Chat-Cre)5.1Deis, RRRC#00658. Rat Resource & Research Center, Columbia, MO, USA). This rat line has a bacterial artificial chromosome (BAC, RP23-246B12) randomly inserted into its genome. This BAC contains the mouse ChAT gene with a Cre insertion before the ATG of the ChAT promoter. This strain is estimated to carry six copies of the transgene (Witten et al., 2011) and was maintained by breeding a carrier male with a wild-type (WT) Long Evans female. This model allowed us to achieve cell-type specificity by using viral vectors dependent on Cre recombinase for expression in order to create either an optogenetic or chemogenetic model of the cholinergic system depending on the Cre-dependent viral vector employed.

Animals were genotyped at age 4-6 weeks via tissue punches taken from the ear and performing polymerase chain reaction (PCR). Procedure and primers are described by Witten et al., (2011). Briefly, we used the Extract-N-Amp Tissue PCR Kit (Sigma Aldrich, Oakville, Ont, CAN) for Cre detection with the primers Cre-F: AAGAACCTGATGGACATGTTCAGGGATCG and Cre-R: CCACCGTCAGTACGTGAGATATCTTTAACC.

Animals were cared for according to the ethical guidelines of the University of Western Ontario Animal Use Subcommittee and Canadian Council on Animal Care (CCAC). For the first 8-

10 weeks of age, animals were group housed. Following surgery, animals were individually housed. Rats were given *ad libitum* food and water, and maintained on a 12 h light-dark cycle. All testing occurred during the light phase.

4.2.2 Validation of Transgenic Model

Our first aim was to ensure our transgenic rat model was appropriate for sensory filtering and sensorimotor gating testing. For this validation both sexes were used and compared with WT littermates as controls (WT n=16, 8 male, 8 female; Cre-ChAT n=16, 7 male, 9 female).

4.2.2.1 Testing of the Acoustic Startle Response

Testing of the ASR was completed in an enclosed sound attenuated startle box from MED Associates (MED-ASR-PRO1, St Albans, VT, USA). Transgenic and WT animals were placed into small transparent tubes (25 cm x 12 cm) mounted on a movement sensitive platform. A piezoelectric transducer mounted below the platform converted the vertical displacement of the platform induced by a startle response into a voltage signal. Startle amplitude was determined using the amplitude of the largest positive and negative peaks of the signal measured in a 300 ms window after the presentation of the acoustic stimulus. Determination of the amplitude was done by MED Associates' software (Startle Reflex version 6.0, MED Associates, Inc. St Albans, VT, USA)

For a schematic representation of ASR testing, see figure 4.1. Testing began with acclimatization to the startle box for 5 minutes every day, for 3 days. During this acclimation, and throughout each test session, there was a 65 dB SPL white-noise background sound. Every testing session began with a 5 min acclimation period. On day 3 rats were tested on an input/output

(I/O) function which assessed startle reactivity. This test began with the presentation of a 65 dB SPL white noise (20 ms) and increased to 120 dB SPL, in 5 dB SPL steps at an intertrial interval (ITI) of 20 s. Startle reactivity determined the setting of the gain of the movement sensitive platform (for detail see Valsamis and Schmid, 2011). This gain amplified the signal from the platform in order to ensure animals startled within a detectable range. Once an animal's gain was determined, it was kept constant throughout the remaining days of testing. The I/O was completed at a gain of 1 for all animals. Following the I/O, experimental testing began.

Experimental testing consisted of two blocks of trials. Block I was used to assess short-term habituation and block two was used to assess PPI. The STH block consisted of 30 startle-alone trials (20 ms white noise, 105 dB SPL, 30 s ITI). The PPI block consisted of 7 different trial types (10 trials/condition, total=70 trials, pseudorandomized order). The trial conditions were as follows: startle pulse-alone trials (for comparison of startle amplitude with prepulse) and combinations of two different prepulses (75 or 85 dB SPL white noise; 4 ms) at three different interstimulus intervals (ISIs; 15, 30 or 100 ms). The ITI for this block varied between 15-25 ms.

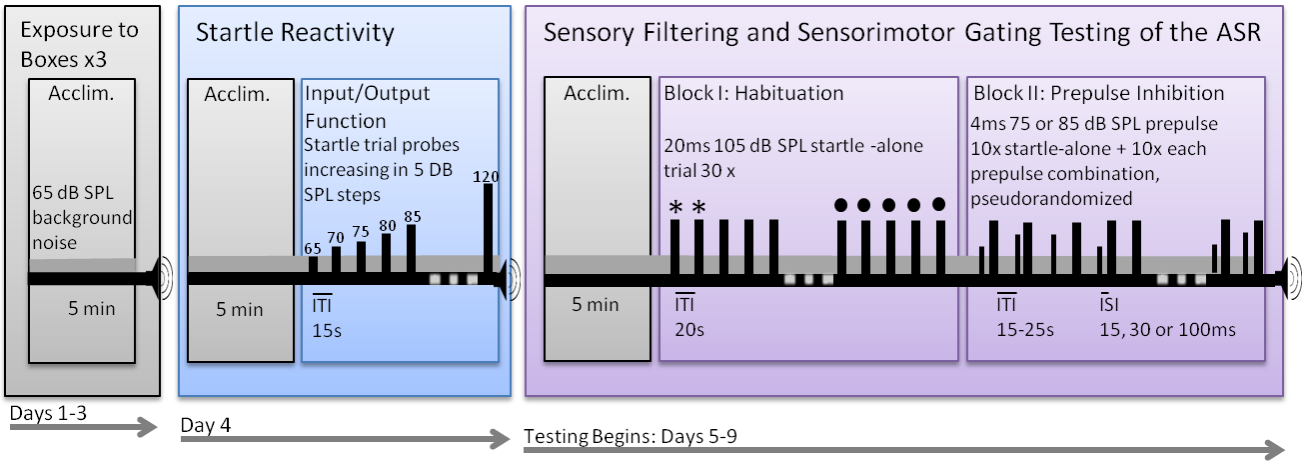

Figure 4.1 Schematic Representation of Startle Testing

Animals were acclimated to the startle chambers three times (indicated by the grey box in the diagram). Animals then completed an input/output function to assess startle reactivity (blue box). During this, animals were presented with increasingly louder auditory stimuli (indicated by the black bars) and the resulting startle magnitude was recorded. Following this, ASR testing began (purple box). Animals began testing with a 5 min re-acclimation to the box. The first block of testing assessed habituation. Identical startle-pulses were presented repeatedly and startle magnitude was recorded. Short-term habituation ratios were calculated by using the average of the final five trials (denoted by dots) divided by the first two startle responses in Block I (denoted by asterisks). Block II assessed PPI which contained a mix of trial types as we used 2 prepulse intensities (75 or 86 dB SPL) and a variety of interstimulus intervals (ISI; 15, 30 or 100 ms). Pseudorandomly placed within this block were 10 startle-alone trials, which were used for quantification of PPI.

4.2.2.2 Statistical Analysis for Habituation and PPI

To analyse the majority of data, we used an analysis of variance (ANOVA). For all statistical analysis included in this chapter, whenever this test was performed, Levene's Test of Equality of Error Variance or Mauchley's Test of Sphericity (repeated measures ANOVA only) was examined. If Levene's Test was violated, an equivalent non-parametric ANOVA was run. In the case of a repeated measures ANOVA, if sphericity was violated, corrections were applied based on the epsilon value (if $\varepsilon<0.75$ the Greenhouse-Geisser correction was applied, or if $\varepsilon>0.75$ the Huynh-Feldt correction was used). If post-hoc tests were necessary, we used student t-tests with Bonferroni corrections. For all analyses included in this chapter, outliers were removed. Outliers were defined as any data point that was outside the range of ±3 standard deviations from the mean (calculated using all animals). Finally, criterion for significance was $\alpha=0.05$.

With regards to ASR testing, in order to detect differences in startle reactivity and startle magnitude between genotypes, we examined the I/O function (where all animals had the same gain factor). We used a three-way repeated measure ANOVA (genotype × sex × sound level) for the I/O function. To analyse short-term habituation of startle, we calculated STH ratios by dividing the average of trials 25-30 by the average of trials 1-2 for each animal; see figure 4.1, (stimuli marked with squares and asterisks respectively) and compared them using a two-way ANOVA (genotype × sex).

Prepulse Inhibition was expressed as percent of prepulse inhibition: %PPI= (1− [startle magnitude with prepulse/baseline startle without prepulse] × 100). This indicated the amount that startle was inhibited, as a percentage of the baseline response. We determined the average

%PPI for each prepulse type and performed four-way ANOVA (prepulse dB SPL × ISI × genotype × sex).

4.2.2.3 Testing of Locomotor Behaviour

We tested the locomotor behaviour of transgenic and WT littermates. To ensure that there was no gross motor impairments in the transgenic model. Rats were placed into a 45 x 45 cm box with 2 adjacent opaque walls and 2 adjacent translucent walls. This occluded the rat's ability to observe other animals during testing. The animals were able to freely explore the box for 20 min while they were tracked using a webcam and ANYmaze software (Version 4.99, Stoelting, Wood Dale, IL, USA). Distance travelled and time spent in the center (6x6 cm centered square) and surrounding perimeter was recorded. The distance each rat travelled and time spent in the center and perimeter of the box were totalled across the 20 min of testing. Additionally, the data for each animal was averaged in to 5 min blocks (total of 4 blocks) in order to assess the changes in locomotor behaviour across time.

To assess short-term habituation of locomotor behaviour a three-way repeated measures ANOVA (genotype × sex × time) was performed. The total distance travelled was analysed using a two-way ANOVA (genotype × sex) and the time spent in the center or perimeter was analysed separately from distance using a repeated measures three-way ANOVA (genotype × sex × area).

4.2.3 Inhibition of Cholinergic PPT Neurons

To inhibit cholinergic neurons of the PPT we induced expression of the designer receptor exclusively activated by designer drugs (DREADD) protein hM4Di, a modified muscarinic receptor, using Cre-dependant viruses in our transgenic (Cre-ChAT) rat model. This receptor is activated

by the biologically inert ligand clozapine N-oxide (CNO). Activation of the hM4Di protein hyperpolarizes neurons through G-protein activation of inward rectifying potassium channels (Armbruster et al., 2007) as well as by inhibiting presynaptic neurotransmitter release (Stachniak et al., 2014)

We performed sensory filtering and sensorimotor gating assessment of the ASR (hM4Di n=12/group, 7 male, 5 female) or exploratory behaviour (n=6/group, males only) following PPT cholinergic inhibition. As a positive behavioural control paradigm, we added assessment of morphine-induced conditioned place preference (CPP; n=6/group, males only). To verify inhibition of cholinergic PPT neurons we completed immunohistochemisty in addition to *in vitro* patch clamp (single neuron analysis) and *in vivo* (multi-unit analysis) electrophysiology.

4.2.3.1 Surgical Procedure

For viral injections into the PPT, animals (aged 10-14 weeks) were induced with a 5% isoflurane and 95% oxygen for induction, and maintained with a 2% isoflurane and 98% oxygen combination. A subcutaneous injection of meloxicam (1 mg/kg) and intramuscular injection of Baytril (10 mg/kg) were administered before surgery and as needed 7 days post-surgery for pain management. Blunt-ended ear bars and a snout mask were used to secure the head in the stereotaxic frame. A midline incision was made in the skin on top of the head, and bilateral bore holes were drilled at the following co-ordinates from bregma: ± 2.0mm medial/laterally, -7.2mm ventrally and either -7.6 (weight 300g) or 7.8mm (weight <400g) caudally. (Paxinos and Watson, 2005). We injected 1 µl/side of virus solution (rAAV8-hSyn-DIO-hDM4(Gi)-mCherry, $5.3*10e^{12}$ vg/ml, Lot: AV4680b. UNC Vector Core, Chapel Hill, NC, USA; or its control rAAV8-hSyn-DIO-

mcherry, 3.8*10e^{12} vg/ml, Lot: AV4680b. UNC Vector Core, Chapel Hill, NC, USA) at a rate of 0.1µl/min using a blunt end 1.0 µl Hamilton syringe (Model 7001 KH SYR, Knurled Hub NDL, 25 gauge, 2.75 in, point style 3; Hamilton, Reno, NV, USA). The syringe rested for 1 min prior to injection and 7 minutes post before retraction. Silk suture was used to close the wound and rats were given a 21 day recovery period to promote maximal expression of the DREADD (or control) protein before testing began.

4.2.3.2 Behavioural Testing With DREADDs

After a 21 day recovery period animals started acclimatization to the startle boxes, underwent I/O testing, and behavioural testing began. Animals were administered an intraperitoneal (IP) injection of the DREADD ligand CNO (10 mg/kg in 18% Dimethyl Sulfoxide (DMSO), Toronto Research Chemicals, Toronto, ON, CAN) or vehicle (18% DMSO, in saline) 20 min prior to testing. Each animal underwent at least 4 days of testing (2 CNO, 2 vehicle). Order of drug administration was randomized and counterbalanced across groups.

Experimental parameters of startle testing were identical to that depicted in figure 4.1, with the exception of a variable ITI (15-25ms) in the habituation block. For a schematic representation of the timeline and testing see figure 4.2. With respect to %PPI, STH ratios and startle reactivity, data was analyzed identically to that described above. Statistical analysis was also similar to that described previously however it now replaced genotype with virus type and included the variable of drug type (CNO vs. vehicle) in ANOVAs (e.g. virus type × drug × sex × prepulse dB SPL × ISI).

Once animals completed startle testing a subset of males (n=6/group), were given a 5 minute rest period in their home-cages before being placed in locomotor boxes (refer to figure 4.2). Administration of CNO was given prior to startle testing which lasted an estimated 30 minutes. In mice, the half-life of CNO is an estimated 1.5 hours (Guettier et al., 2009) but behavioural effects in rats have been shown to persist much longer (Mahler et al., 2014; Wirtshafter and Stratford, 2016), therefore cholinergic PPT inhibition was still in effect during locomotor testing. Experimental parameters were identical to those discussed previously, see section 4.2.2.3. Data analysis was similar to that described above, however, genotype was replaced with virus type and included the variable of drug type (CNO vs. Vehicle) in ANOVAs (e.g. virus type × drug × time).

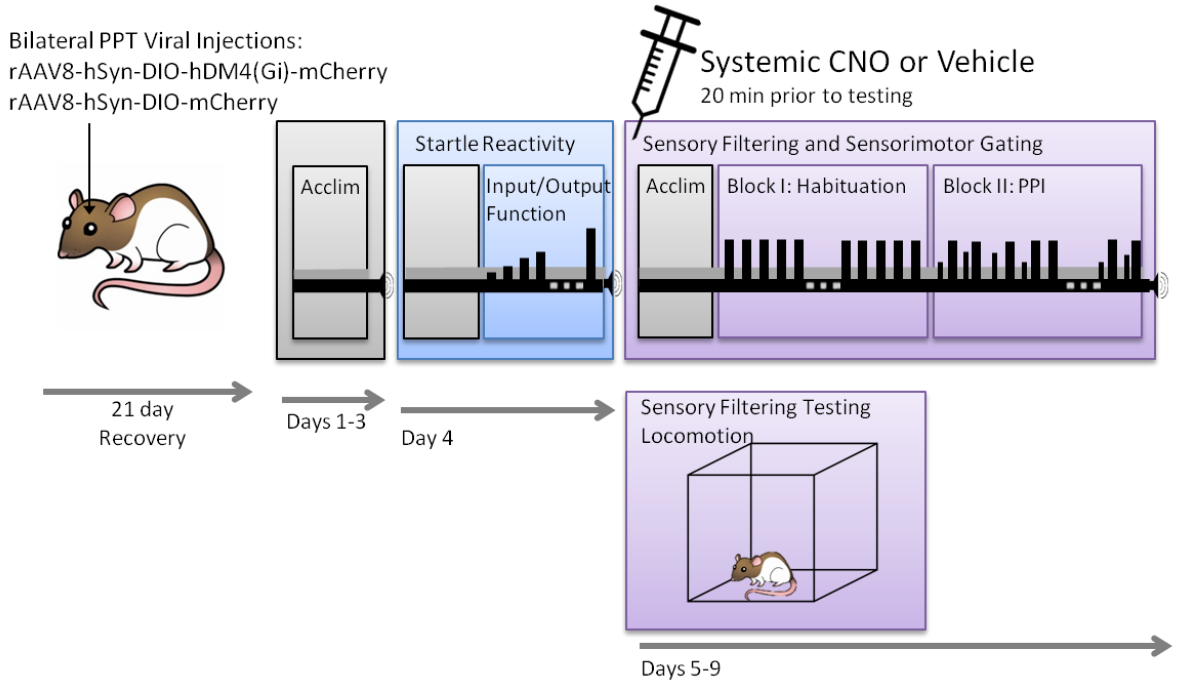

Figure 4.2 Behavioural Testing During DREADD Inhibition of Cholinergic PPT Neurons

Prior to testing, animals received bilateral infusions of a Cre-dependent virus (hM4Di or mCherry control) into the PPT. 21 days later, behavioural assessment began. Animals were first acclimated to the startle chamber three times (5 min, grey box). Their startle reactivity was then assessed using an input/output function. After this, habituation and PPI of the ASR was tested. This testing was roughly the same as described in figure 4.1, except there was a variable ITI of 15-25 s during ASR testing. Additionally, testing was preceded with either a systemic injection of CNO (10 mg/kg in 18% DMSO) or vehicle (18% DMSO) 20 min prior to testing. Following ASR testing there was a 5 min rest in their home cages before animals completed locomotor testing.

4.2.3.3 Conditioned Place Preference (Positive Behaviour Control)

A subset of males (n=6/group) underwent an unbiased, counterbalanced, conditioned place preference (CPP) procedure, as described previously (Ahmad et al., 2013). Briefly, saline vehicle or morphine injections (5 mg/kg IP, administered immediately prior to placement in the chamber) were paired with one of two environments that differed in color (black or white), texture (smooth floor or textured with woodchip bedding), and smell (2% acetic acid or no added scent). As reported previously, rats display no baseline preference for either of these two environments (Laviolette and van der Kooy, 2003). Animals received alternating exposure to the morphine-paired and saline-paired environment for a total of 3 exposures per environment (1 session/day). Drug assignment to environment type was counterbalanced. Thirty minutes prior to the morphine or saline injection, all animals were administered CNO (10 mg/kg in 18% DMSO, IP).

Three days after the conditioning phase ended, animals were tested for a place preference (with no injections prior to testing). They were able to freely explore a test chamber for 10 min. The test chamber consisted of two compartments with environments identical to those used in conditioning on either end with a small neutral gray zone separating them. Testing began when the animal was placed in this neutral zone and the distance travelled and time spent in each compartment was recorded and tracked using a webcam and ANYmaze software (Version 4.99, Stoelting, Wood Dale, IL, USA). For each animal, CPP behavior was expressed by plotting the time spent in each compartment and using an individually calculated place preference score (time in morphine paired environment/time in saline).

Time spent in each chamber during the test day was analyzed using a repeated measures ANOVA (environment × virus). The preference score was analysed using a one-sample t-test. As no preference would have a score of 1, we used a one-tailed t-test for each group to determine if this score significantly differed from no preference.

4.2.3.4 Immunohistochemistry

Animals were euthanized by IP injections of an overdose of sodium pentobarbital (Euthanyl: Bimeda-MTC Animal Health Inc. Cambridge, ON, CAN) and transcardially perfused with saline followed by 4% paraformaldehyde. The brains were harvested and stored in 30% sucrose until sliced into 40 µm slices using a freezing microtome (KS34S, Thermo Fischer Scientific, Waltham, MA, USA). Slices were divided into 4 parallel series and stored at -20˚C in cryo-protectant solution (30% sucrose, 30% ethylene glycol, and 5% of 0.01% sodium azide in 0.1M phosphate buffer (PB)).

Prior to free-floating immunohistochemistry being performed, as well as in between all incubations with antibodies, all slices were thoroughly rinsed in 0.1M PB. Antibodies were delivered in a 0.1M PB and 0.1% bovine serum albumin (BSA) solution. Slices were pre-treated with a 1% H_2O_2 in 0.1M PB (Caledon Laboratories Ltd., Georgetown, ON, CAN) for 10 min then blocked for 1 hour in a 0.1M PB plus 0.4% Triton X-100 and 0.1% BSA (Fisher Scientific, Hampton, NH, USA) solution before incubation with primary antibodies. Next, tissue was incubated overnight with an anti-mCherry antibody (1:25000, rabbit, polyclonal; ab167453 Abcam, Toronto, ON, CAN). The mCherry antibody was amplified, first by incubating tissue with a goat anti-mouse biotinylated secondary antibody (1 h, 1:500; Vector Laboratories, CA, USA), followed by avidin-

biotin complex solution (1h, 1:500; Vectastain Elite ABC Kit, Vector Laboratories, Burlington, CA, USA). Finally, slices were treated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (0.04% H_2O_2, 0.2mg/ml DAB; Sigma-Aldrich, Oakville, ON, CAN).

To label cholinergic neurons, slices were then incubated in β nicotinamide adenine dinucleotide phosphate (NADPh) solution (0.3% Triton-X, 0.1 mg/ml nitroblue tetrazolium, 1 mg/ml β NADPh, Sigma-Aldrich, ON, Canada) and rinsed. Tissue was then mounted onto positively-charged glass slides and cover-slipped.

The tissue was imaged using a bright field Nikon DS-Qi2 microscope (Nikon, NY, USA) and associated NIS-Elements AR software. Images were taken at 2 and 20x objectives.

4.2.3.4.1 Co-expression of mCherry and the Cholinergic Marker NADPh

Images were taken for each animal between post-surgery days 26-35. For each animal, a representative image was taken at the injection site as well as one posterior and anterior to the site at 20x magnification. The number of blue (NADPh), brown (mCherry), and blue and brown (co-expressed) cells were counted manually by two individuals. Cell counts were tracked using ImageJ software using the Fiji cell counter plugin (Schindelin et al., 2012; Schneider et al., 2012). The inter-rater reliability was calculated using a two-way mixed effects model intra-class correlation (ICC) and was revealed to be strong (ICC: 0.93). Once counted, an average of the two cell counts was used for reporting and analysis. This method has been adapted from Pitchers et al., (2010).

4.2.3.5 *In Vitro* Patch Clamp Electrophysiology

In a separate group of animals, 0.3 µl of AAV virus containing mCherry (n=2) or hM4Di (n=4) were injected bilaterally into PPT of 5 week old transgenic Cre-ChAT rats. Rats were anesthetized with isoflurane 21-26 days after surgery and their brains were extracted. Brains were cut into 300 µm coronal slices using a vibrating-blade microtome (HM 650V, Thermo Fischer Scientific, Waltham, MA, USA) in ice-cold carbogen-equilibrated solution containing (in mM): 2.5 KCl, 10 $MgS0_4$, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 0.5 $CaCl_2$-$2H_2O$, 11 Glucose, and 234 Sucrose. Slices containing the PPT (Bregma -7.20 mm to -8.10 mm) were then incubated at 32°C for 1 hour in artificial cerebrospinal fluid (ASCF) containing (in mM): 124 NaCl, 3 KCl, 3 $MgS0_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $CaCl_2$-$2H_2O$ and 10 Glucose.

4.2.3.5.1 Patch Clamp Recordings

Electrophysiological experiments were performed at room temperature and the protocol was similar to that of previous studies (Zaman et al., 2011; Zaman et al., 2014). In K^+-based whole-cell current clamp mode, the spontaneous firing properties of PPT mCherry-positive neurons were recorded in ASCF bubbled with carbogen.

Cholinergic neurons expressing the Cre-dependent viral marker, mCherry, were visualized using an upright microscope (Zeiss Axioskop, Germany), equipped with an EMCCD camera (Evolve 512, Photometric, Tuscon, AZ). Recording electrodes were pulled on a P-97 Puller (Sutter Instrument, Novato, CA, USA) from fabricated borosilicate glass capillaries (1B150F-4, OD;1.50 mm, ID;0.84 mm, World Precision Instruments, Sarasota, FL, USA) and had 4–6 MΩ tip resistance when filled with an intracellular solution containing (in mM): 140 K-gluconate, 10 KCl, 1 $MgCl_2$, 10 HEPES, 0.02 EGTA, 3 Mg-ATP, and 0.5 Na-GTP, pH adjusted to 7.35, 290–300 mosm/l.

Slices were treated with 5 µM CNO (in 0.1% DMSO) for 10 minutes in recording chamber. The spontaneous activity before and after the CNO treatment was recorded in PPT neurons at -50 mV holding potential. Signals were sampled at 10 kHz, amplified with Axopatch 200B, digitized with Digidata-1550 and analyzed using pClamp10.4 (all Axon Instruments, Molecular Devices, Sunnyvale, CA, USA).

To analyse the spontaneous firing frequency of mCherry-positive PPT neurons pre- and post-CNO treatment, we performed a two way repeated measures ANOVA (virus type × drug).

4.2.3.6 *In Vivo* Electrophysiology

A total of 9 animals were used to assess the efficacy of hM4Di-induced inhibition *in vivo* (hM4Di n=4, 2F, 2M mCherry n=5, 3F 2M). Animals were anesthetized using an initial dose of ketamine (80 mg/kg IP) and xylazine (5 mg/kg), and supplemental doses were given intramuscularly as needed. The rat's head was fixed in a stereotaxic frame using blunt ear bars and a headpost was attached to the skull with acrylic dental cement. A stainless steel screw was inserted into the right frontal skull bone to serve as an anchor for the headpost and electrical ground. A craniotomy (2 x 2 mm; 5-8 mm posterior to bregma) was performed in the left frontal skull bone to expose the brain. At the end of the surgical procedure, the right ear bar was removed to allow free-field auditory stimulation during the electrophysiological recordings in the contralateral PPT.

4.2.3.6.1 *In vivo* Recordings

Extracellular electrophysiological signals were collected using a 32-channel electrode array which consisted of a single shank with 32 equally-spaced recording sites, spanning 0.25 mm

in length (A1x32-Poly3-10mm-25s-177-A32; NeuroNexus Technologies, Ann Arbor, MI). The electrode array was connected to a high-impedance headstage (NN32AC, Tucker Davis Technologies (TDT), FL, USA), and the neuronal activity was pre-amplified and digitized (two RA16SD Medusa preamps, TDT). It was then sent to a RZ5 processing module. For each of the 32 channels, the neuronal activity was digitally sampled at 25 kHz and bandpass filtered online at 300 – 3000 Hz using a voltage threshold for spike detection of three standard deviations above the noise floor.

To record, the electrode was inserted in the brain 7.6-7.8 mm posterior from bregma and 2.0 mm medially from bregma. Using a hydraulic microdrive (FHC, ME, USA), the electrode array was lowered until all 32 recording sites were within the PPT (estimated depth of 7.2-7.7 mm from the skull). The depth of penetration was determined by probing the auditory responsiveness of brain regions and mapping their progressive changes with increasing depth. Once the electrode was determined to be in the PPT, it settled for 1 hour before conducting recordings. The electrode was coated with DiI to verify placement.

In each subject, an audio stimulation paradigm was performed before and 20 min post an administration of CNO (10 mg/kg in 18% DMSO, IP via a butterfly catheter inserted prior to the rat being placed in the stereotactic frame). To assess auditory-evoked responses in the PPT, computer-triggered auditory stimuli were presented using a RZ6 processing module (TDT, 100 kHz sampling rate) and custom Matlab software. Auditory stimuli consisted of noise bursts (1-32 kHz; 50 ms duration) from a speaker (MF1; TDT) positioned 10 cm above the surface of the

stereotaxic frame and 10 cm from the base of the right pinna on a 30 degree angle from midline in the contralateral space. Auditory stimuli were 85 dB SPL (4 ms, white noise bursts).

4.2.3.6.2 Offline Sorting and Statistical Analysis

To analyse multi-unit data, custom scripts in Matlab were used to generate rasters and Peri-Stimulus Time Histograms (PSTH). The average spontaneous activity of a multi-unit cluster was determined using the average firing rate within the final 100 ms of each trial. Auditory responsiveness was determined as the average firing rate across trials within a 2-12 ms window of time after the auditory stimulus onset. For representative rastor plots and PSTHs, see supplemental figure A.2. We defined a multi-unit cluster to as responsive to an auditory stimulus if it displayed a significantly increased firing rate during auditory stimulation compared to spontaneous activity, as determined using paired t-test.

Multi-unit activity of spontaneous and auditory-evoked activity was compared prior to CNO administration to 20 min post CNO administration. The average percent change in spontaneous and auditory-evoked firing rate was calculated for each multi-unit cluster (e.g. %Change= Activity pre-CNO/Activity post-CNO x 100%). This was then averaged for each animal and within each virus type. For each animal, we also determined the proportion of multi-units who's activity decreased (%Change <0) and averaged this within groups. Both these measures were compared between groups using an unpaired Student's t-test.

4.2.3.6.3 Immunohistochemisty and Imaging

Immunohistochemistry was performed to verify electrode placement and virus expression similarly to described in section 4.2.3.4, however because the electrode was coated

with a fluorescent marker, DiI, we used a fluorescent secondary antibody for mCherry fluorescently (Alexa-594, donkey anti-rabbit, 1:500 Thermo-Fischer Scientific Waltham, MA, USA).

Images were acquired using a Leica LSM 800 (Zeiss, Germany) confocal microscope and associated Zen software (Zeiss, Germany) using 10x magnification. Images were scanned using the 546 nm laser line and collected wavelengths included 550-700 nm.

4.2.4 Optogenetic Activation of Cholinergic PPT Neurons

To activate cholinergic neurons of the PPT we induced expression of the optogenetic protein, a modified Channel Rhodopsin 2 (ChR2(H134R)) using Cre-dependent viruses in our transgenic (Chat-Cre) rat model. The light-gated ChR2(H134R) ion channel is activated maximally by blue light (465 nm), and once opened cations freely enter the cell according to electrochemical gradients. The ChR2(H134R) opsin has been modified to conduct a larger current compared to ChR2 when activated, although it displays slightly slower protein kinetics (Fenno et al., 2011).

We completed sensorimotor gating assessment of the ASR paired with photostimulation of the PPT (ChR2(H134R) n=7, 1M 6F YFP n=6, 1M 5F). The same animals also completed optogenetically-induced CPP testing as a positive behavioural control. To further verify that our photostimulation paradigm was effective in activating cholinergic neurons in the PPT, animals received photostimulation 60-90 min prior to perfusion, and c-FOS expression was analysed.

4.2.4.1 Surgical Procedure

To induce expression of the light-sensitive channel ChR2(H134R) we bilaterally injected the Cre dependant rAAV5-EF1α-DIO-hChR2(H134R)-eYFP ($4.3*10e^{12}$ vg/ml, Lot:AV4313p. UNC

Vector Core, Chapel Hill, NC, USA) or its control rAAV5-EF1α-DIO-eYFP ($4.9*10e^{12}$ vg/ml, Lot:AV4836c. UNC Vector Core, Chapel Hill, NC, USA). Viruses were aliquoted and stored at -80 °C. For more details regarding surgical procedure and coordinates see section 4.2.3.1.

We injected 1.0 µl of virus per side, at a rate of 0.1µl/min. The syringe rested for 7 min before retracting. Bilateral fiber-optic cannulae (7.2 mm, 400/430 µm core, NA 0.48; Doric Lenses, Franquet, QC, CAN) were then lowered into the same location where viral injections had taken place. Once in place, they were secured using acrylic dental cement. Three jeweler's screws were placed in the skull to improve security of the implants (2 bilaterally over the parietal skull bones and 1 on the left frontal skull bone). Silk suture was used to close the wound and rats were given a 28 day recovery period to promote maximal expression of ChR2(H134R) before testing.

4.2.4.2 Optogenetic Stimulation During Sensorimotor Gating Assessment

28 days post-surgery animals began ASR testing (ChR2(H134R) n=7, 1M 6F; YFP n=6, 1M 5F). First, they were acclimated to a modified startle chamber: the startle platform and speakers were identical to that explained in the previous section, however animals were tested in a clear, rectangular holding chamber (w: 25 cm, h: 30 cm), placed in a partially enclosed box. This ensured the rat was comfortably tethered to the Light Emitting Diode (LED; housed with a fiber-optic rotary joint: FRJ 1x1, Doric Lenses, Franquet, QC, CAN), which was suspended on top of the box. The LED was connected to the animal using a branching opto-patchcord (400/430 µm core, 0.48 NA, Doric Lenses, Franquet, QC, CAN).

Prior to testing, animals were acclimated to the tether procedure. Animals were tethered two times (15 min) while freely exploring their home cages. During their first exposure to the

startle chambers, animals were not tethered (15 min), however for the following 3 acclimation procedures they were tethered to the LED but received no photostimulation. Since tethering required the use of a larger holding chamber during startle testing, the acclimation procedure was lengthened to 15 min to reduce movement artifacts during testing.

An I/O function identical to that described previously (section 4.2.2.1) was done to determine startle reactivity. Once testing began, it was composed of three blocks of trials (figure 4.3). The first block consisted of 20 habituation trials (105 dB SPL white noise, 20 ms duration), with a variable ITI of 20-60 s. This was to ensure startle magnitude was relatively stable before optogenetic manipulations.

The second block was to determine the effects of PPT cholinergic optogenetic stimulation on baseline startle. It consisted of 15 trials (105 dB SPL white noise, 20 ms duration) with a fixed ITI of 60 s. The first 5 trials were startle-alone trials. The next 10 startle stimuli were presented with concurrent optogenetic stimulation (photostimulation duration: 60 ms, see below section 4.2.4.2.1 for details, triggered 1 ms prior to startle pulse, duration: 20 ms). To determine the impact of concurrent photostimulation on startle we normalized trials with photostimulation to the average 3 trials that preceded photostimulation for each individual rat. Additionally, we averaged trials with photostimulation and those without and divided these values to create an individualized total ratio (Stim Ratio: avg with stim/avg without). This was compared between groups using a one-way ANOVA.

Block III assessed auditory PPI and optogenetically-induced PPI. The first trial consisted of an auditory prepulse (85 dB SPL, 4 ms) with an ISI of 15, 30, 100 or 200 ms. In the following trial

blue light was delivered to the PPT (causing activation of these cells) instead of the auditory prepulse. These trial types were termed Opto-induced PPI. Prepulse inhibition was analysed using a three way ANOVA (virus type × prepulse type × ISI).

4.2.4.2.1 Photostimulation

Optogenetic stimulation was triggered by a 28V signal from the Med Associates boxes, which was transformed using a converter (SG231, Med Associates Inc.) into a TTL-pulse. This TTL-pulse triggered a waveform generator (DG1022, Rigol Technologies, OR, USA) which was used to modulate light stimulation. The waveform generator triggered the LED driver (LED RV 1Ch 1000 Single LED, Doric Lenses, Franquet, QC, CAN), which controlled the LED light fiber.

Light stimulation was delivered using a blue LED (465 nm, FRJ 1x1, Doric Lenses, Franquet, QC, CAN), CAN) at 50 Hz (3 pulses of 15 ms of light, 5 ms rest), 10 Hz (3 pulses of 15 ms, 85 ms rest), or 1 Hz (15 ms pulse). Light illumination varied from 21-24.7 mW. Optical power was measured using an energy meter console (PM100D paired with photodiode sensor S120C, Thorlabs Inc., NJ, USA).

In a subset of experimental animals (n=3, 1 male, 2 female), testing was re-run with unilateral stimulation of the anatomical left PPT, or low-light stimulation of 1-4 mW/side (data reported in appendix A). There was a minimum of 2 days separating testing. Data analysis was similar to that explained previously, but we introduced the photostimulation (frequency or laterality) and into the ANOVA (virus type × stimulation × ISI).

4.2.4.2.2 Drugs

All animals were re-tested with the 50 Hz photostimulation as described above, but received an IP injection of mecamylamine hydrochloride (3 mg/kg) or saline, 7 days apart, prior to testing. During startle testing, the acclimation was shortened to 7 min to account for the half-life of mecamylamine (1.2 hours, Debruyne et al., 2003). Short-term habituation curves, ratios and PPI were calculated as described above (section 4.2.2.2). To analyze PPI however, a separate repeated measures ANOVA (virus × drug × ISI) was performed for photostimulation as a prepulse and auditory PPI.

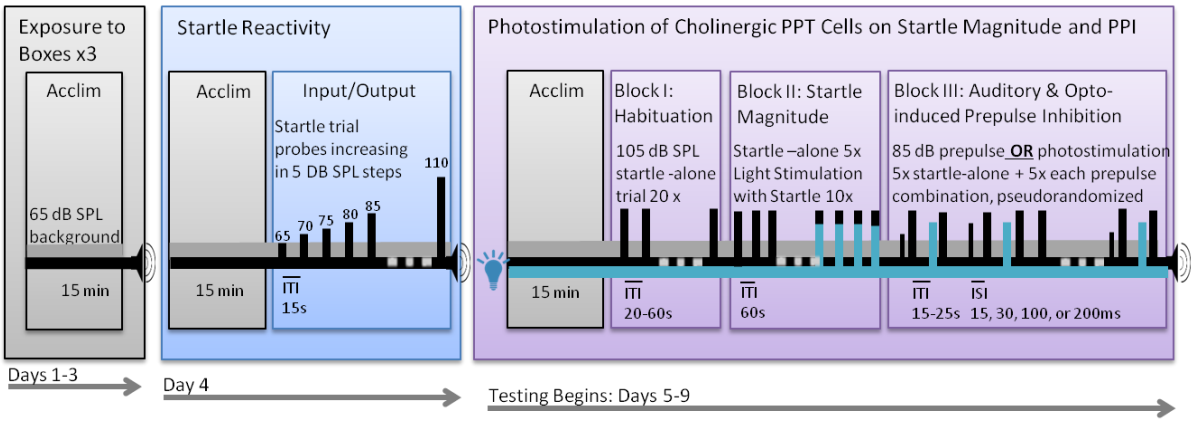

Figure 4.3 Schematic of Sensorimotor Gating Testing Paired with Optogenetic Stimulation
Animals were acclimated to the startle chamber three times (indicated by the grey box). Animals then completed an input/output function to assess startle reactivity (blue box) where animals were presented with increasingly louder auditory stimuli (indicated by the black bars) and the resulting startle magnitude was recorded. Animals began testing with a 5 min re-acclimation to the box. Block I habituated the animal so that startle reached a stable level of responding. Block II assessed the role of cholinergic release from the PPT on startle magnitude. Photostimulation of the PPT coincided with the presentation of a startle pulse. Photostimulation is represented by the blue bar. In the final block of testing (block III), some prepulses were replaced by photostimulation to optogenetically-induce PPI. Prior to a startle-pulse, animals were presented with an auditory prepulse OR photostimulation. Pseudorandomly placed within this block were 10 startle alone trials to determine baseline startle amplitudes.

4.3.2.3 Opto-Induced CPP

The same rats were used as in startle experiments above (n=7, 1 male, 6 female ChR2; YFP-control n=6, 1 male, 5 female). The general CPP procedure has been described above. Instead of pairing a chamber type with morphine, here we paired it photostimulation of cholinergic PPT neurons (50 Hz, 25 x 15 ms light pulse; 19.8-22.3 mW). Animals were placed in either chamber type for 30 minutes for a total of 3 exposures to each environment type. Photostimulation-paired and unpaired chamber environments were counterbalanced. Animals were tested the next day. Testing parameters and data analysis occurred in the same manner described previously (section 4.2.3.3). During testing animal were tethered, but did not receive light stimulation.

4.3.2.4 Immunohistochemistry and Imaging

Prior to perfusion, animals received bilateral 50 Hz light stimulation (25 pulses of 15 ms duration/min) for 30 min. Animals were perfused 60-90 min after, as previously described. Immunohistochemistry was performed using an antibody for c-FOS (1:1000; polyclonal rabbit, Sc-52, Santa Cruz Biotechnology, Dallas, TX, USA) and a secondary antibody Alexaflour594 (donkey anti-rabbit, ThermoFischer Scientific, Waltham, MA, USA), in order to ensure stimulation parameters activated target neurons. This validation of photostimulation has been used in the past (see Liu et al., 2012; Yamamoto et al., 2015). In order to verify virus expression in cholinergic cells we used an antibody for choline transporter (ChT; 1:500, monoclonal mouse, EMD Millipore, Etobicoke, ONT, CAN) and amplified this using the ABC method described previously and tagged this using Streptavidin Alexaflour594 conjugate (1:1000, ThermoFischer Scientific, Waltham, MA, USA). Staining was identical to that described previously. Co-expression with the fluorescent tag EYFP, labelling neurons expressing the ChR2(H134R) protein, was analyzed. Images were

acquired using a Leica LSM 800 (Zeiss, Germany) confocal microscope using 20 & 40x magnification. Images were scanned using the 488 and 546 nm laser lines individually, and we collected wavelengths 490-550 and 560-700 nm, respectively. Images were merged using Zen software (Zeiss, Germany). Analysis of images was described previously (section 4.2.3.5). To estimate cellular activation with photostimulation, two individuals counted the number of yellow fluorescent protein (YFP)-expressing neurons, and c-FOS positive cells, as well as the number of YFP neurons that co-expressed c-FOS for both experimental (ChR2(H134R)-YFP) animals and controls (YFP only). Cell counts were tracked using ImageJ software using the Fiji cell counter plug-in (Schindelin et al., 2012; Schneider et al., 2012). The inter-rater reliability between counters was calculated using a two-way mixed effects model ICC which revealed an acceptable correlation (0.90). Once counted, an average of the two cell counts was used for reporting.

4.3 Results

The results of this chapter are broken into three sections. The first examines if the Cre-ChAT transgenic ratline is a valid model to use for sensory filtering and sensorimotor gating testing. The second examines the effects of inhibition of cholinergic PPT neurons on these processes, whereas the final section examines the effect of activation of these neurons. For all these experiments, both male and female rodents were used; while this factor was always included in our statistical analysis (except for optogenetic experiments), we only graphed groups according to sex when it was determined to significantly influence our results.

4.3.1 Validation of Cre-ChAT Transgenic Rats as an Appropriate Model

In order to achieve cell-type specificity we used a transgenic rodent model (Long Evans-Tg(Chat-Cre)5.1Deis). This animal model was generated using a BAC containing the genomic sequence for the Cre protein right after the ChAT promoter (Witten et al., 2011). Using this model allowed us to use Cre dependent viruses to target solely cholinergic cells. Transgenic mice previously created with this BAC have a greatly enhanced cholinergic tone which has been shown to alter cognitive function (Kolisnyk et al., 2013). This was due to the BAC which contained the open reading frame for the vesicular acetylcholine transporter (VAChT) protein between the first and second exon of the ChAT gene. VAChT is the rate limiting factor for ACh release, so over-expression of VAChT may increase ACh release. Therefore, it was important to investigate if sensory filtering and sensorimotor gating processes were normal in transgenic rats.

4.3.1.1 Startle Reactivity in the Cre-ChAT Transgenic Rat Model

Prior to habituation and PPI testing, transgenic rats (Cre-ChAT n=16, 7 male, 9 females) and WT littermates (n=16, 8 males, 8 females) had their startle reactivity assessed using an I/O function. This function documented the changes (if any) in startle magnitude with increased auditory stimulus intensity (65-120 dB SPL).

A three way ANOVA (sex × genotype × sound level) revealed that both genotypes showed increasing startle magnitudes in response to increasing sound intensities (Greenhouse-Geisser corrections applied; $F_{(3.6,97.2)}$=29.6, p<0.001). Most importantly, both genotypes began to startle at the same stimulus level (85 dB SPL, see figure 4.4A), however transgenic animals reached a maximum startle magnitude that was lower than that of WT littermates. While there was no significant effect of sex ($F_{(1,27)}$=2.2, p=0.15) or genotype ($F_{(1,27)}$=2.2, p=0.15), and no significant

interaction between genotype, sex, and sound intensity ($F_{(3.6,97.2)}$=2.4, p=0.23), there was a significant sex by genotype interaction ($F_{(1,27)}$=5.6, p=0.03). As shown in figure 4.4B, WT males showed a greater maximal startle magnitude compared to female counterparts ($F_{(1,14)}$=6.4, p=0.02). This sex difference was absent in Cre-ChAT animals ($F_{(1,13)}$=0.5, p=0.49). Differences in weight, which could influence the range of signal detection of our startle measurements, did not contribute to this finding, as both sexes' weights were similar across genotypes (WT female: 280g ±5, Cre female: 275g ±6g, WT male: 455g ±14, Cre male: 453g ±8).

However, as Cre-ChAT rats of both sexes showed increased startle magnitudes with increasing stimulus intensity, had a similar startle threshold at around 80-85 dB SPL, and reached a maximal startle amplitude that was both robust and at similar sound levels to that of WT females, we believed their startle reactivity was still appropriate for sensory filtering testing.

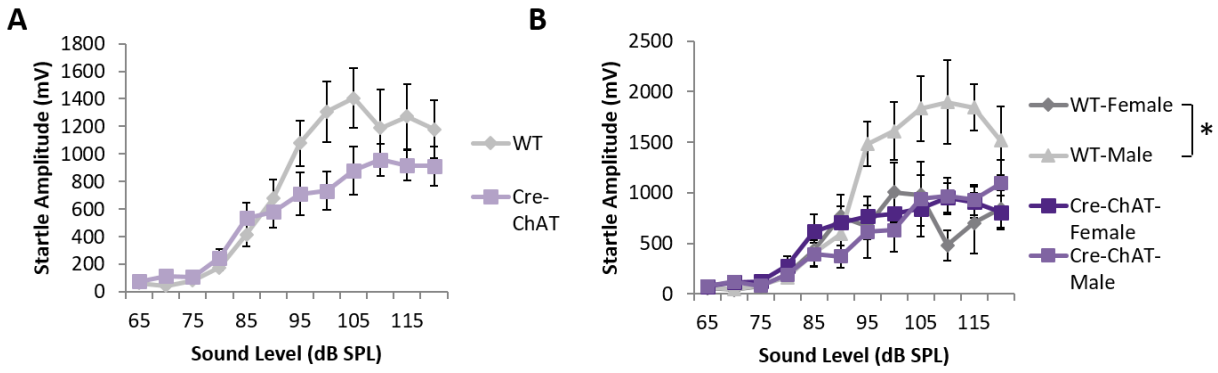

Figure 4.4 Startle Reactivity of Transgenic Cre-ChAT Rats Compared to Wild-type Littermates
As shown in A), both genotypes increased their startle amplitude with increasing sound intensity. Additionally, both genotypes began to display a robust startle response at the same stimulus level (~80-85 dB SPL), however the transgenic animals reached a maximum startle magnitude that was lower than that of wild-type. Further analysis determined that this was due to sex differences. As

shown in B) WT males and females show a difference in maximal startle amplitude, which is absent in Cre-ChAT rats (Cre-ChAT n=16, 7 males 9 females, WT n=16, 8 males, 8 females).

4.3.1.2 Short-Term Habituation of the ASR in the Cre-ChAT Transgenic Rat Model

Startle amplitude was plotted across trials in order to examine short-term habituation of the ASR (figure 4.5A). This was analyzed using a three way repeated measures ANOVA (genotype × sex × trial) which revealed that both genotypes showed progressively decreased startle magnitudes as there was a significant effect of trial ($F_{(29,754)}$=6.11, p<0.001). There was no impact of genotype ($F_{(1,26)}$=0.2, p=0.66) or sex ($F_{(1,26)}$=1.18, p=0.29), and no interaction between trial, genotype, and sex ($F_{(29,754)}$=1.03, p=0.43) which indicated that the rate of habituation in transgenic rats was not different from WT.

We quantified the total reduction in startle magnitude using a short-term habituation (STH) Ratio. This reflected the final startle amplitude relative to the initial startle. As shown in figure 4.5B, both genotypes displayed equal ratios ($F_{(1,26)}$=2.34, p=0.13), with WT showing reduced responses to 0.65 (±0.08) of initial magnitude and Cre-ChAT rats to 0.55 (±0.05). In contrast to the analysis above, which examined the progression of habituation, we found a significant effect of sex ($F_{(1,26)}$=0.22, p=0.64) when we compared the habituation score. This effect was equal in both genotypes as there was no sex by genotype interaction ($F_{(1,26)}$=0.41, p=0.53) or effect of genotype ($F_{(1,26)}$=2.3, p=0.14). In both cases, males showed significantly more short-term habituation, as shown in figure 4.5C.

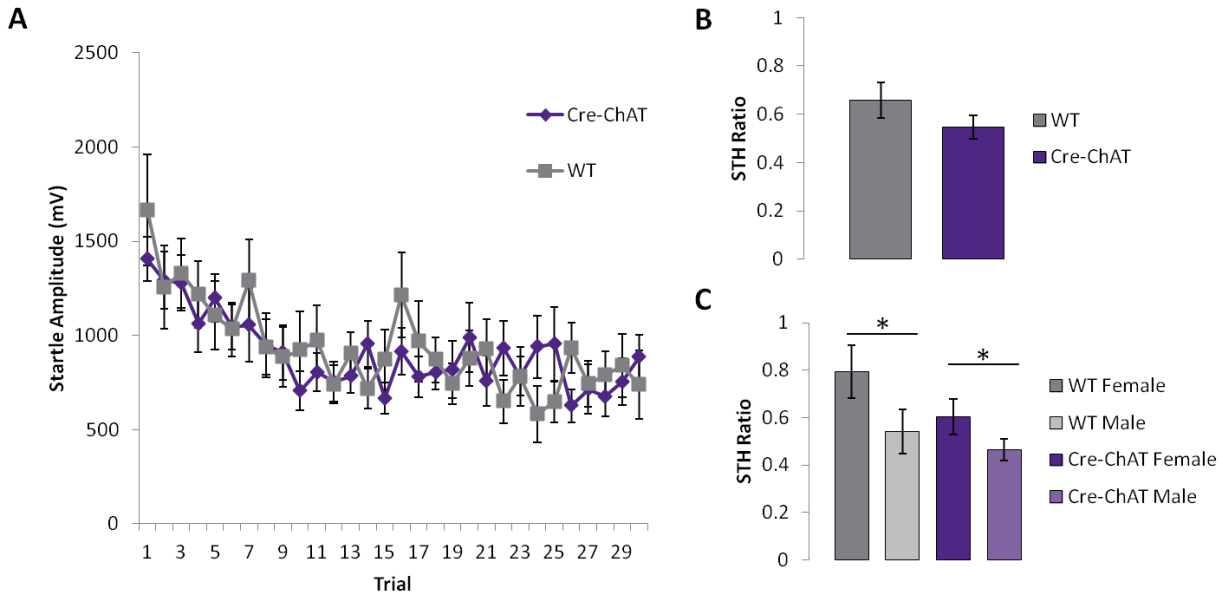

Figure 4.5 Short-Term Habituation of the ASR is Unaltered in Cre-ChAT Rats

A) The progressive decrease in startle amplitude across trials in both genotypes was not different. The total reduction in startle amplitude for each animal was normalized using an STH Ratio (B &C). Both genotypes showed decreased startle to ~0.55-0.65 of initial response magnitude (shown in B), however, in both genotypes, the different sexes displayed significantly different amounts of habituation (shown in C). In general, males tended to show stronger reduction of startle (Cre-ChAT n=16, 7 males, 9 females, WT n=16, 8 males, 8 females).

4.3.1.3 Short-Term Habituation of Locomotor Behaviour in the Cre-ChAT Transgenic Rat Model

We found no difference in the short-term habituation of locomotion or locomotor behaviour of transgenic Cre-ChAT rats (figure 4.6). The distance travelled during 5 minute blocks of open field testing was analysed using a three way repeated measures ANOVA (genotype × sex × time). Both groups showed decreased activity during the 20 min exploration as we observed a significant effect of time ($F_{(3,81)}$=224.8, p<0.001), indicative of short-term habituation. Both genotypes travelled to a similar degree as we saw no effect of genotype ($F_{(1,27)}$= 0.1, p=0.75). We did uncover a significant effect of sex ($F_{(1,27)}$=5.3, p=0.03) but no interaction between sex, genotype, and time ($F_{(1,27)}$=3.8, p=0.07), which indicates that the influence of sex was equal across genotypes.

To further uncover the effect of sex, we calculated the cumulative distance travelled by each genotype and sex throughout the duration of the test and examined this using a two-way ANOVA (sex × genotype). As shown in figure 4.6B females travelled to a greater degree than their male counterparts in both genotypes ($F_{(1,27)}$=10.4, p<0.01).

To further ensure transgenic rats displayed normal open field behaviour, we calculated the total time spent in the perimeter versus the center of the open field (figure 4.6C). This ratio is also indicative of anxiety-like behaviour, since anxious animals tend to stay closer to the walls. Consistent with previous studies (Lamprea et al., 2008), we documented that both genotypes displayed a strong preference for the perimeter of the open-field (three way repeated measures ANOVA sex × genotype × area: $F_{(1,27)}$=522, p<0.001). This was equal across genotypes ($F_{(1,27)}$=0.1, p=0.75) and sexes ($F_{(1,27)}$=0.14, p=0.71), and there was no interaction between any of these

factors ($F_{(1,27)}$=1.3, p=0.26). Overall this revealed that locomotor activity of the transgenic rats was not different from WT animals.

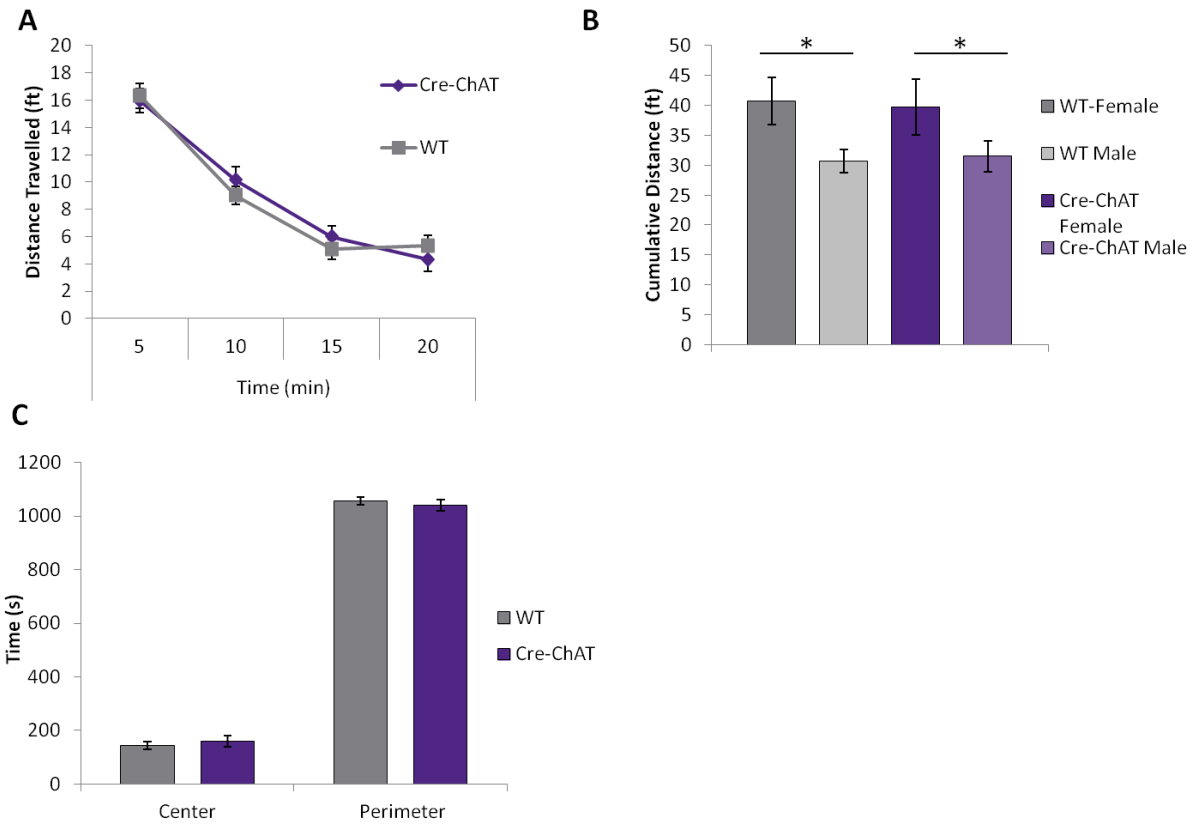

Figure 4.6 Short-Term Habituation of Locomotor Behaviour in Cre-ChAT Rats

A) Short-term habituation of exploratory behaviour was not different between WT and transgenic rats. B) Overall, female rats were significantly more active than males (B), but that this was equal across genotypes. C) Transgenic rats displayed the same strong preference for the perimeter as WT littermates, indicative of normal thigmotactic behaviour (Cre-ChAT n=16, 7 males, 9 females, WT n=16, 8 males, 8 females).

4.3.1.4 Prepulse Inhibition of the ASR in the Cre-ChAT Transgenic Rat Model

As our hypothesis was mainly centered upon sensorimotor gating of the ASR, it was critical to ensure that PPI was normal in Cre-Chat transgenic rats. Displayed in figure 4.7 is the percent of inhibition induced by a prepulse (%PPI) across genotypes. A four way ANOVA (genotype × sex × prepulse dB SPL × ISI) showed that both genotypes showed similar startle inhibition by a prepulse as we detected no main effect of genotype ($F_{(1,27)}$=0.03, p=0.86) or sex ($F_{(1,27)}$=0.12, p=0.73), or interaction between genotype and sex ($F_{(1,27)}$=0.91, p=0.34).

In general, we saw PPI of 65-79% in both genotypes (depending on ISI). Using a 75 or 85 dB SPL prepulse resulted in similar PPI ($F_{(1,27)}$=0.6, p=0.44), however at both prepulse levels, we saw a significant difference according to ISI ($F_{(2,54)}$=6.3, p<0.01). We tended to observe maximal PPI using the 30 ms ISI (see figure 4.7A).

Baseline startle during the PPI block of testing was not different between genotypes (two way ANOVA genotype × sex: $F_{(1,27)}$=0.72, p=0.40), or sex ($F_{(1,27)}$=0.28, p=0.60). There was also no interaction between group and sex on baseline startle amplitude ($F_{(1,27)}$=0.47, p=0.5). Baseline startle was measured using individually calibrated movement-sensitive platform (gain values; see section 4.2.2.1). Together, this data implies that PPI was not different between transgenic and WT animals (figure 4.7B).

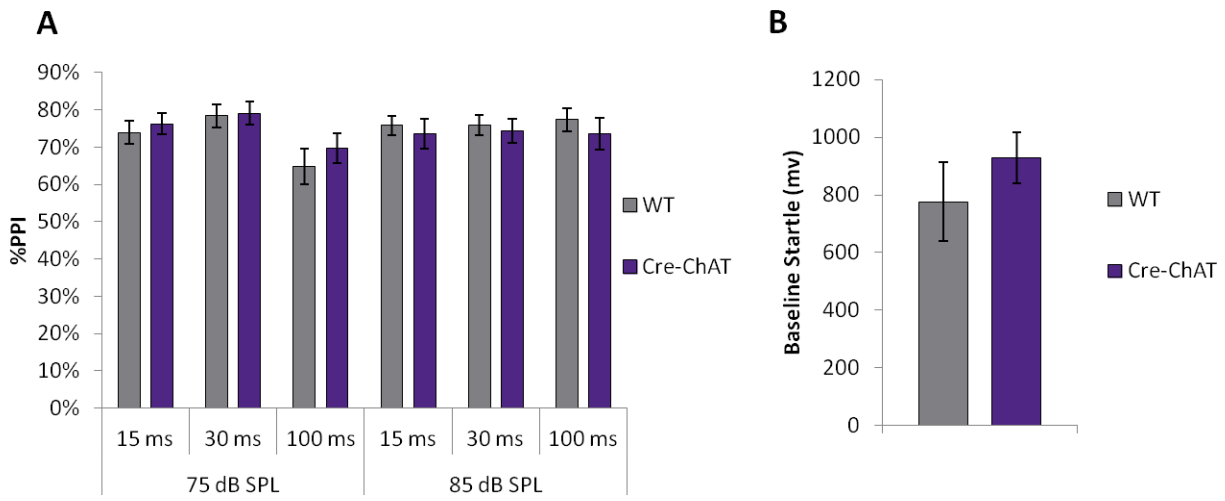

Figure 4.7 Prepulse Inhibition is Normal in Cre-ChAT Transgenic Rats

A) The amount of PPI in transgenic and WT rats. The data is plotted as %PPI, which reflects the amount of inhibition relative to baseline startle amplitude. Both genotypes showed similar startle inhibition by a prepulse. B) Baseline startle magnitude (calculated from startle pulse alone trials during PPI measurements) of WT and transgenic animals showed no difference between genotypes (Cre-ChAT n=16, 7males, 9 females, WT n=16, 8 males, 8 females).

4.3.1.5 Summary of Behavioural Validation of the Cre-ChAT Transgenic Rat Model

Overall, we observed that transgenic rats had similar startle reactivity, short-term habituation and PPI of the ASR as their WT littermates. Additionally, locomotor activity and short-term habituation of non-reflexive behaviours were not significantly altered in this rat model. Therefore, I concluded that these rodents were an appropriate model to use for the remainder of our studies.

4.3.2 DREADD-Induced Inhibition of Cholinergic PPT Neurons

For chemogenetic inhibition of cholinergic PPT neurons we injected a Cre-dependent DREADD virus (rAAV8-hSyn-DIO-hM4Di-mCherry; n=12, 7 males, 5 females) or control virus (rAAV8-hSyn-DIO-mCherry; n=12, 7 males, 5 females) into the PPT of Cre-ChAT rats. Animals were tested using a 10 mg/kg dose of CNO (IP, 18% DMSO) or vehicle. This dose of CNO was chosen based on pilot data (Appendix A).

4.3.2.1 Startle Reactivity of hM4Di Expressing Rats Compared to mCherry Controls

Prior to conducting startle testing, all animals completed an I/O function. There was no administration of CNO or vehicle prior to this, so we included data from our pilot group (n=16/group, 7 females, 9 males). We used a 3-way repeated measures ANOVA (virus × sex × dB SPL) to analyze unamplified startle amplitudes.

Both groups showed an increase in startle magnitude as sound level increased ($F_{(6.6,178)}$=26.5, p<0.001). Surprisingly, we observed a significant effect of virus ($F_{(1,27)}$=8.0, p=0.01), but no effect of sex ($F_{(1,27)}$=1.4, p=0.31). As clearly shown in figure 4.8, startle magnitude of the hM4Di expressing animals was consistently greater than mCherry expressing control animals. As

we found no interaction between virus type, sex, and sound intensity ($F_{(6.6,178)}$=0.6, p=0.73), which suggested that there was greater startle responses in hM4Di animals, but that the relative increase in this response with sound intensity was equal to that of controls. In other words, the startle-reactivity curve between each virus group progressed similarly, but the hM4Di curve reached a higher maximum level.

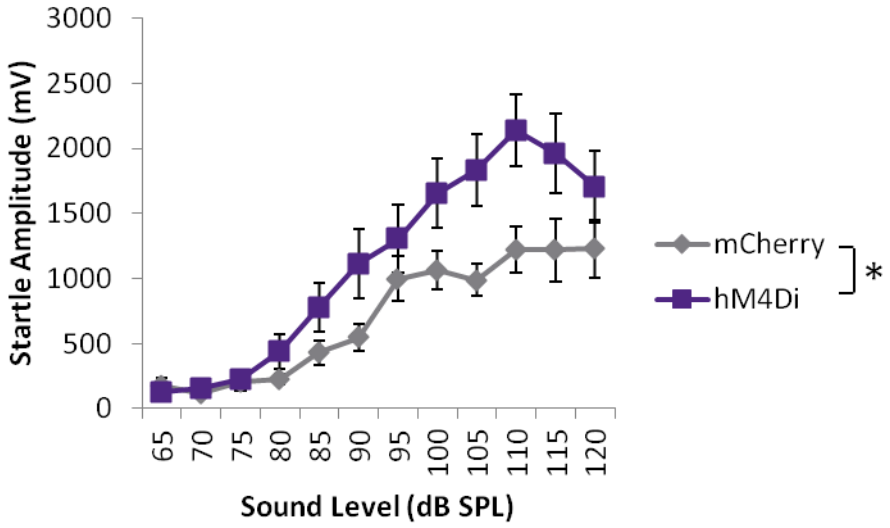

Figure 4.8 Startle Reactivity in hM4Di Expressing Animals is Altered

Both groups of transgenic animals showed increased startle magnitudes with increasing sound intensity; however the hM4Di expressing animals, on average, displayed significantly higher startle amplitudes, while startle threshold and sound level for maximum startle amplitude remained the same. It is important to note that this test was run without CNO administration (n=16/group, 7 females, 9 males).

4.3.2.2 Short-term Habituation of the ASR Following Inhibition of Cholinergic Neurons of the PPT

In order to quantify habituation, startle amplitudes were normalized to the average of the first two trials for each animal. This helped to remove the differences in absolute startle reactivity observed between individual animals and virus types (see figure 4.8), while also reducing the impact of any long-term habituation that may occur across testing sessions. One female rat from each group (mCherry and hM4Di) was removed from this analysis as they were outliers (STH ratio: ±3 standard deviations). Animals received CNO (10 mg/kg IP) or vehicle (18% DMSO) prior to testing. As revealed by the repeated measures ANOVA (virus × sex × drug x trial), both hM4Di and mCherry control animals showed progressively decreased startle across trials, indicative of short-term habituation ($F_{(29,522)}$=4.4, p<0.001). There was no main effect of sex ($F_{(1,18)}$=0.9, p=0.35), or drug ($F_{(1,18)}$=0.74, p=0.78); however there was a significant effect of virus ($F_{(1,18)}$=4.90, p=0.04), as well as a significant interaction between virus, drug and trial ($F_{(29,522)}$=1.8, p<0.01). A follow up repeated measures ANOVA (trial × sex × drug) within each group revealed that CNO administration significantly impacted the short-term habituation in hM4Di expressing animals ($F_{(1,9)}$=5.90, p=0.04), but not in mCherry controls ($F_{(1,9)}$=1.33, p=0.27).

As shown in figure 4.9A, it appeared that CNO administration improved short-term habituation in the hM4Di expressing animals. This was further quantified using STH Ratios, as shown in figure 4.9B. In control animals startle responses were reduced to about half of their initial magnitude when given vehicle or CNO (0.53 ± 0.06, 0.52 ± 0.08, respectively). With vehicle administration, hM4Di expressing animals showed decreased responding by the same amount (0.53 ± 0.09), however, when given CNO, this ratio decreased to 0.37 (±0.05) which indicated

improved habituation. A repeated measures ANOVA (sex × virus × drug) on STH ratios revealed that this trend failed to reach statistical significance, as we saw no effect of drug ($F_{(1,18)}$=1.90 p=0.19), virus ($F_{(1,18)}$=1.05, p=0.32), or sex ($F_{(1,18)}$=1.77, p=0.2), and no interaction these factors on STH ratios ($F_{(1,18)}$=0.78, p=0.39).

Overall, this suggests that inhibition of cholinergic PPT neurons altered short-term habituation as our ANOVAs revealed that the habituation curve of hM4Di animals was significantly changed when given CNO. During cholinergic PPT inhibition, hM4Di animals showed a greater and more prolonged gradual decrement in startle before reaching a stable level of responding (by around trial 20) compared to control groups (figure 4.9A). This also resulted in a slightly lower STH ratio in the hM4Di animals when given CNO (figure 4.9B), however, the latter failed to reach statistical significance. Thus, it appeared that the greatest effect of DREADD-inhibition was detected in the slope of the STH curve, which reflects a faster rate of habituation.

4.3.2.3 Short-term Habituation of Locomotion Following Inhibition of Cholinergic Neurons of the PPT

For analysis of locomotion only male rats were used (n=6/group). We saw normal short-term habituation in exploratory behaviour in both mCherry and hM4Di expressing rats. Ambulatory distance decreased greatly across time, as revealed by a three way repeated measures ANOVA (time × drug × virus). There was a significant effect of time (Greenhouse-Geisser corrections applied: $F_{(2.3,20)}$=67.8, p<0.001), and at each time point, both groups travelled to a similar degree as there was no effect of virus type ($F_{(1,9)}$=0.2, p=0.67), or CNO administration ($F_{(1,9)}$=0.9, p=0.37), and no interaction between these factors ($F_{(5,45)}$=0.6, p=0.7). Overall, this

suggests that inhibition of the cholinergic PPT neurons do not influence the short-term habituation of non-reflexive behaviours, as displayed in figure 4.9C.

As an important control we calculated the cumulative distance travelled, shown in figure 4.9D, and analysed this using a two way ANOVA (virus × drug). We found that total distance travelled did not differ between virus groups ($F_{(1,9)}$=0.1, p=0.76). We observed no main effect of drug ($F_{(1,9)}$=0.4, p=0.54), or interaction between these factors ($F_{(1,9)}$=4.0, p=0.08), which denoted normal activity levels after cholinergic PPT inhibition.

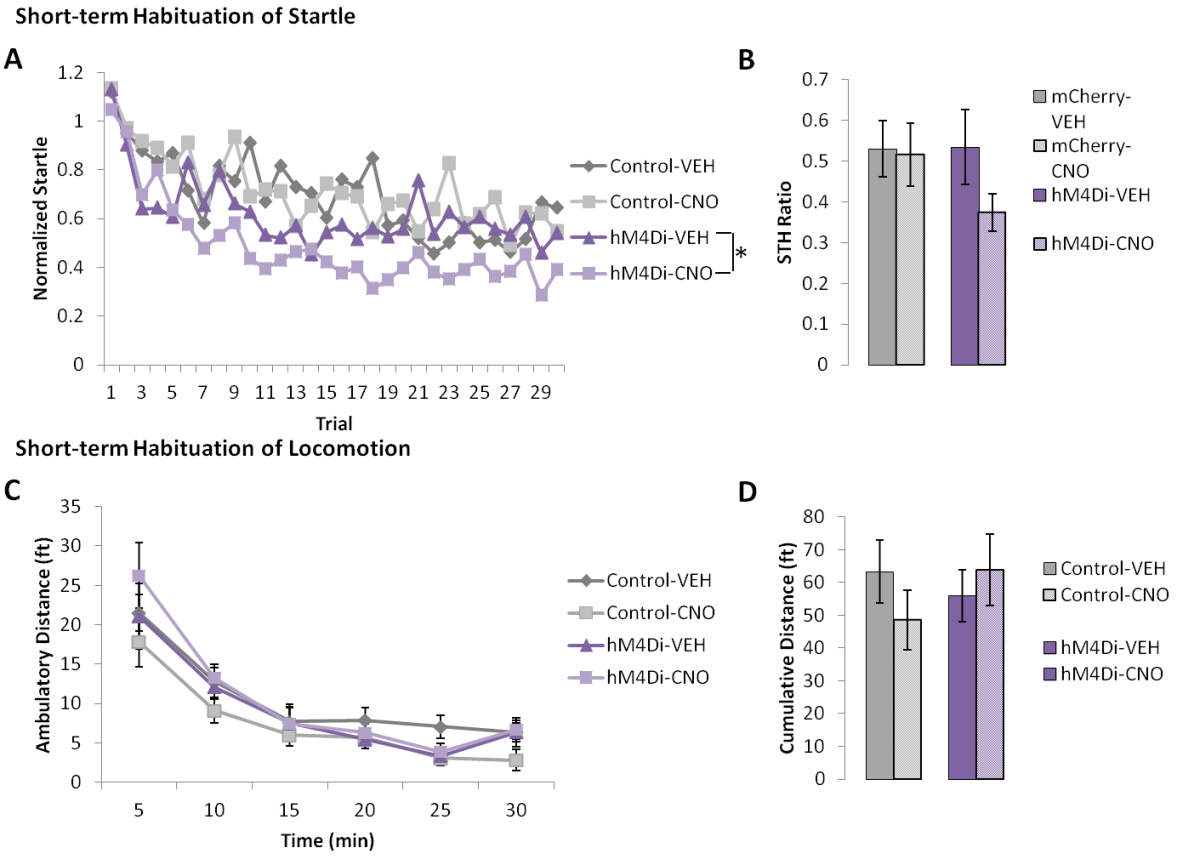

Figure 4.9 Cholinergic Inhibition of the PPT Improves Short-Term Habituation of the ASR but not Locomotion

A) All groups showed short-term habituation of startle as there was a progressive decrease in normalized startle magnitude (data normalized to the first 2 trials within each animal). However, there was a significant increase in habituation in hM4Di expressing animals when administered CNO. B) In hM4Di expressing animals there was a trend toward further decreased STH ratios, which failed to reach statistical significance (n=11/group, 7 males, 4 females). C) Both virus groups showed reduced exploration of the open field across time, indicative of normal habituation. This decrease was unaffected by CNO administration. D) Displays total distance travelled, which did not differ between virus groups or with CNO treatment (n=6 males/group).

4.3.2.4 Prepulse Inhibition of the ASR Following Inhibition of Cholinergic Neurons of the PPT

Overall, we did not find an influence on PPI during inhibition of the cholinergic cells of the PPT, in contrast to our initial hypothesis. A repeated measures ANOVA (virus × sex × drug × ISI × dB SPL) on %PPI values revealed no effect of drug ($F_{(1,20)}$=1.1, p=0.3), virus type ($F_{(1,20)}$=0.7, p=0.49) or sex ($F_{(1,20)}$=0.8, p=0.48). Silencing cholinergic PPT neurons did not influence PPI across ISIs or prepulse intensity as we found no interaction between virus, drug, sex, prepulse intensity, and ISI ($F_{(2,40)}$=0.2, p=0.82).

Consistent with past PPI studies, we observed a significant effect of prepulse intensity ($F_{(1,20)}$=11.42, p<0.005), as shown in figure 4.10A, with greater PPI following the more intense prepulse (85 dB SPL). We also observed a significant effect of ISI ($F_{(1,40)}$=9.48, p=0.001), and maximum PPI occurred using a 30 ms ISI. At this ISI (and a 85 dB SPL prepulse), control animals displayed 83% (±4%) inhibition with vehicle administration and 79% (±4%) with CNO; similarly hM4Di expressing animals showed 84% (±2%) inhibition with vehicle and 85% (±2%) with CNO.

As shown in figure 4.10B, we observed that inhibition of cholinergic PPT neurons impacted baseline startle magnitude. Although our three-way repeated measures ANOVA (virus × sex × drug) revealed a trend towards an effect of drug ($F_{(1,20)}$=4.3, p=0.05), we saw no effect of virus, ($F_{(1,20)}$=0.5, p=0.47), sex ($F_{(1,20)}$=0.1, p=0.86), or interaction between drug, sex and virus ($F_{(1,20)}$=1.0, p=0.31). However, we did observe a significant interaction between drug and virus ($F_{(1,20)}$=4.7, p=0.04). Post-hoc t-tests revealed that CNO selectively decreased baseline startle in the hM4Di expressing group (t_{11}=2.6, p=0.02) but not in mCherry controls (t_{11}=0.36, p=0.72).

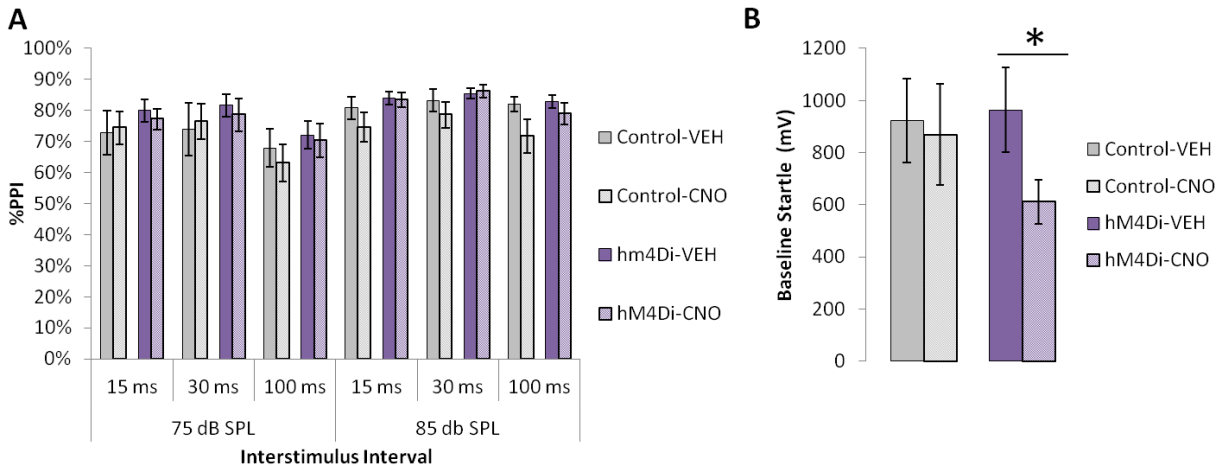

Figure 4.10 Inhibition of Cholinergic PPT Neurons did not Impair Prepulse Inhibition, but did Alter Startle Magnitude

Contrary to the major predictions of the field, inhibiting the cholinergic cells of the PPT did not disrupt PPI, as shown in A). Prepulse inhibition is plotted as % PPI, which reflects the amount of inhibition induced by a prepulse. We saw no difference in %PPI between control expressing mCherry animals and hM4Di infected animals, and no effect of systemic CNO administration across all testing conditions. During PPI testing, we did observe a decrease in baseline startle magnitude selectively in the hM4Di expressing group when given CNO, shown in B). This suggests that cholinergic inhibition of PPT cells may influence startle reactivity (n=12/group, 5 female, 7 male).

4.3.2.5 *In vitro* Patch Clamp Recordings of hM4Di Expressing Neurons

In order to determine the efficacy of our DREADD-induced inhibition on a cellular level, *in vitro* patch clamp recordings of PPT neurons expressing the viral tag mCherry were performed in the current clamp mode (mCherry n=2, hM4Di n=4). Cells were held at a resting membrane potential of -50 mV by a constant current. Prior to CNO administration, firing frequency was 1.96 Hz (±0.11) in mCherry expressing control cells and 1.95 Hz (±0.11) in hM4Di expressing cells. After being bathed in CNO (5 µM in 1% DMSO) the firing frequency of mCherry cells was unchanged (1.96 Hz (±0.21), whereas the firing of hM4Di expressing cells was greatly reduced (0.19 Hz ± 0.06; figure 4.11). This reduction was statistically significant as a two way repeated measures ANOVA (virus × drug) revealed a significant effect of drug ($F_{(1,4)}$=37.04, p=0.004), and interaction between drug and virus ($F_{(1,4)}$=37.56, p=0.004). Overall this shows on the cellular level that CNO effectively inhibits hM4Di expressing neurons.

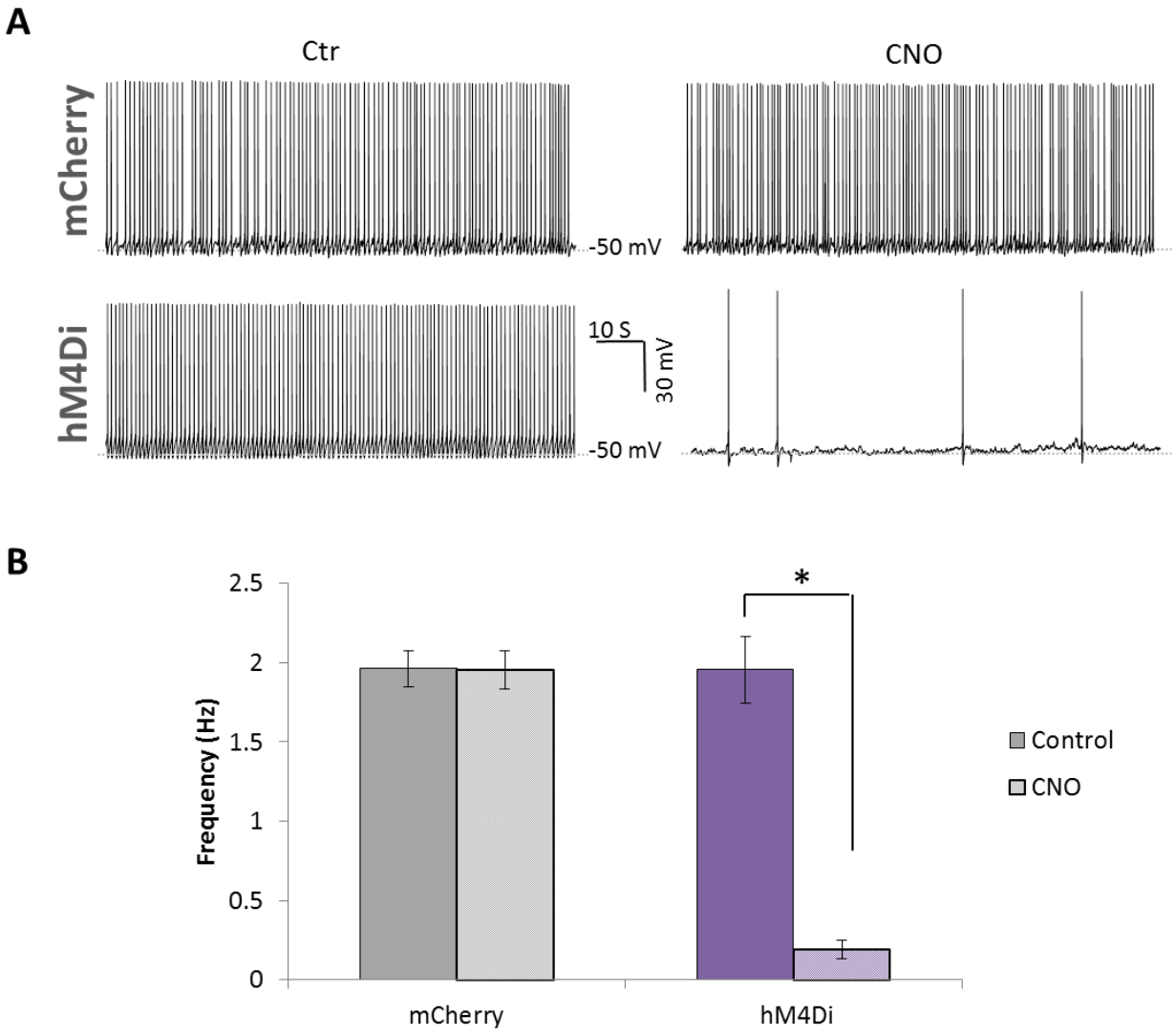

Figure 4.11 hM4Di Expressing Neurons are Strongly Inhibited by CNO *In Vitro*

A) A representative trace of electrophysiological *in vitro* patch clamp recordings of mCherry positive neurons in the PPT in control mCherry and hM4Di-mCherry neurons before and after CNO treatment. B) Both types of neurons showed similar spontaneous firing rates when the membrane potential was held at -50 mV. When treated with CNO, hM4Di neurons showed a drastic decrease in firing frequency, whereas mCherry control neurons were unaffected (mCherry n=2, hm4Di n=4 slices).

4.3.2.6 *In vivo* Electrophysiological Recordings of the PPT Following hM4Di-Induced Inhibition of the Cholinergic PPT Neurons

In order to observe the effects of DREADD-induced inhibition of the cholinergic PPT neurons at the population level, and to determine if this inhibition impacts auditory processing, we performed *in vivo* electrophysiological recordings (mCherry n=5, hM4Di n=4 animals). For each animal, we normalized individual multi-unit cluster activity to pre-CNO levels (%Change=activity with CNO/activity pre-CNO, 100%=no change). As shown in figure 4.12C, we saw that spontaneous activity was slightly, but significantly, decreased after systemic CNO administration in hM4Di animals as their spontaneous firing frequency was 93% (±4) of pre-CNO levels, whereas the mCherry controls firing frequency was 106% (±3; t_6=3.6, p=0.01). Moreover, the proportion of multi-units within each animal that displayed a decrease in activity post-CNO (%Change<0) was also significantly greater in hM4Di animals (70% ±8) compared to mCherry controls (41% ±4; t_6=3.63, p=0.01). This is displayed in figure 4.12D. As we expected to inhibit only a subset of neurons within the PPT, i.e. cholinergic, but not glutamatergic or GABAergic cells, our slight but significant inhibition of multi-unit spontaneous activity confirms our DREADD system is functional at a population level *in vivo*.

Interestingly, the auditory-evoked multi-unit activity to an 85 dB SPL noise burst was unchanged after cholinergic inhibition by CNO administration (t_6=1.7, p=0.14). Control animals' auditory-evoked activity was 103% (±3) of pre-CNO levels, whereas hM4Di animals displayed 95% (±8) of pre-CNO activity. The proportion of auditory-evoked multi-unit responses within each animal that showed a decrease (%Change >0) was not different between groups either (t_6=1.5,

p=0.18), with 40% ± 9.5 in mCherry animals and 57% ±14 in hM4Di animals (figure 4.12E & F). Overall this suggests that auditory responsive cells in the PPT are mainly non-cholinergic.

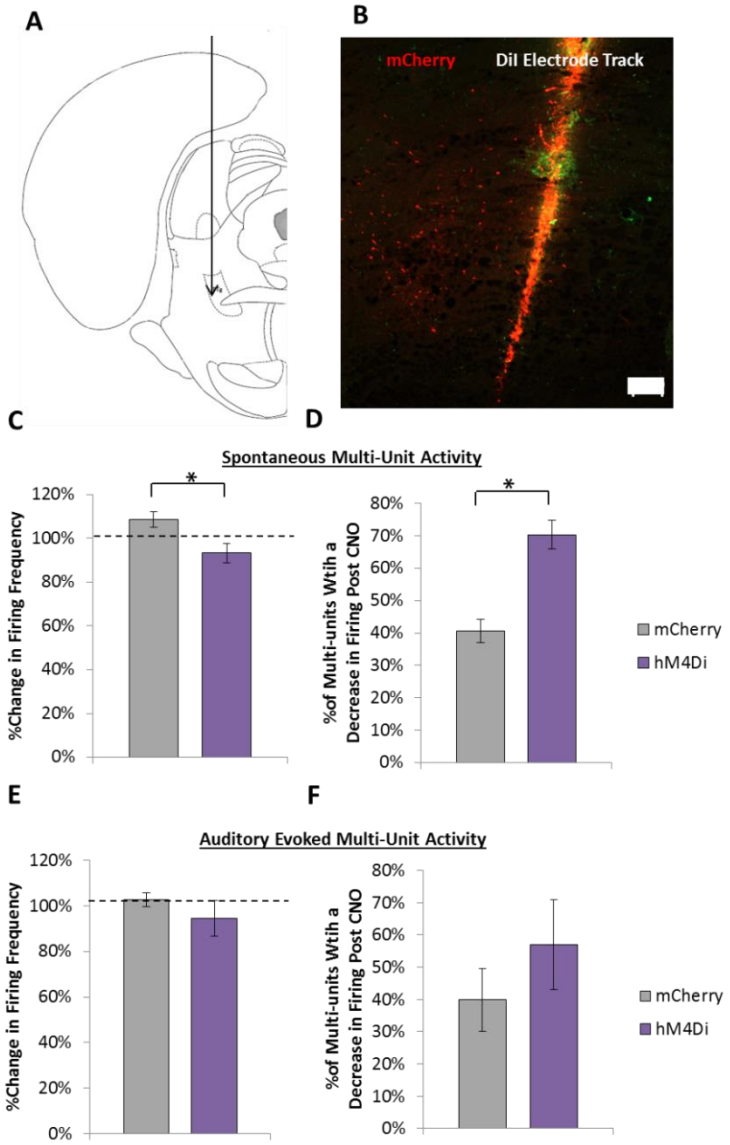

Figure 4.12 The Spontaneous Activity of Multi-Unit Clusters is Reduced in hM4Di Animals via CNO

A) Depicts the target area of recording within the PPT. Image was adapted from Paxinos and Watson (2005). B) The orange trace shows where the recording electrode, coated with DiI, was

placed, red cells are mCherry positive cells. Included in this analysis are animals with recording sites verified within the PPT and close to mCherry positive cells. Scale bar is 200 µm. C) The activity of each multi-unit was normalized to pre-CNO levels (100%, as indicated by the dotted line) and averaged within each animal to create an overall average for each group. The spontaneous activity of multi-units in hM4Di expressing animal's post-CNO was significantly lower than controls (mCherry: 109%, hM4Di: 93%). D) Additionally, the average proportion of multi-units that decreased their activity (%Change <0) was significantly greater in hM4Di animals. E) Relative activity of multi-units to auditory stimulation (85 dB SPL, 4 ms white noise) post CNO (pre-CNO = 100%, as indicated by the dotted line). Auditory responsiveness was not different between groups post CNO. F) The proportion of multi-units that displayed decreased activity (%Change <0) post CNO was also not different between groups. This suggests that auditory responsiveness was intact in hM4Di animals, despite inhibition of cholinergic neurons (hM4Di n=4, mCherry n=5).

4.3.2.7 Conditioned Place Preference Following Cholinergic Inhibition of the PPT (Positive Behavioural Control)

Although the experiments above provide strong evidence that our DREADD system inhibited cholinergic PPT neurons at the cellular and population level, it was important to prove that our inhibition was also able to alter behaviour. Therefore, as a positive behavioural control, we performed morphine-induced CPP. A subset of male rats used in ASR testing were used for CPP testing (n=6/group). Pre-treatment with CNO was given 30 min prior to all conditioning sessions (morphine and saline-paired environments). On the test day the time spent in each chamber was analysed using a three way repeated measures ANOVA (virus × drug × environment type).

We observed that DREADD-induced inhibition of cholinergic neurons disrupted the development of CPP. We found a significant effect of drug ($F_{(1,10)}$=5.9 , p=0.03) and interaction between drug and virus type ($F_{(1,10)}$=5.3, p=0.04). Post hoc t-tests revealed that on average, control animals spent more time exploring a morphine-paired environment compared to the saline-paired (t_5=3.6, p=0.02). There was no place preference in the hM4Di animals (t_5=0.06, p=0.94), as shown in figure 4.13A. We further quantified this using a preference score (time in morphine-paired environment/time in saline; 1=no preference). As shown in figure 4.13B, mCherry expressing control animals displayed a preference score of 3.96 (±0.7) which was significantly greater than one (one sample t-test; t_5=2.9, p=0.02), indicating a strong preference for morphine. Again, this was absent in hM4Di animals who had a score of 1.20 (±0.34; one sample t-test t_5=0.8, p=0.23). Overall this suggests that our DREADD-induced inhibition of cholinergic PPT neurons was effective at preventing the development of CPP.

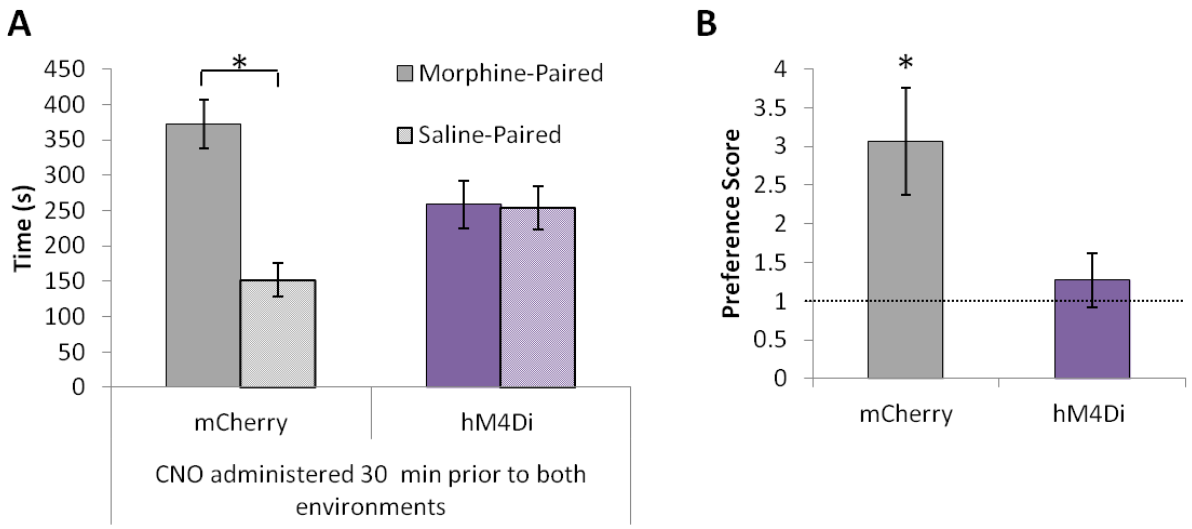

Figure 4.13 Morphine-Induced Conditioned Place Preference is Blocked by Inhibition of Cholinergic PPT Neurons

A) During the conditioning phase, both groups were pre-treated with CNO 20 min prior to exposure to saline- or morphine-paired environments. We observed that inhibition of cholinergic neurons disrupted the development of CPP. On test day hM4Di animals spent equal time in both environments, whereas mCherry controls spent more time in the morphine-paired environment, exhibiting CPP. B) CPP within each animal was quantified using a preference score (time spent in Morphine-paired environment/time in Saline, 1=no preference as indicated by the dotted line). Animals expressing mCherry displayed a preference score significantly greater than 1, but hM4Di animals did not (n=6 males/group).

4.3.2.8 Verification of DREADD Virus Expression

Bilateral expression of the viral-tag mCherry in the PPT was verified for all animals included in this study. Figure 4.14 displays a representative image of virus expression throughout the PPT. We examined mCherry expression within the PPT for five representative slices (see figure 4.14). Then, the proportion of animals expressing mCherry at that representative slice was calculated (n=12 animals total; # of animals expressing mCherry/12). In general, most injections (90%+) targeted the mid to caudal aspect of the PPT, however good expression occurred through the majority of the PPT. In some animals, uni-or-bilateral expression was also observed in the LDT (mCherry n=2, hM4Di n=1) and PGB (mCherry n=4, hM4Di n=5). Overall, expression patterns did not differ substantially between mCherry control animals (data not shown) and hM4Di expressing animals.

To determine the proportion of cholinergic cells targeted by the virus, we performed immunohistochemistry for the viral-tag mCherry and examined the co-expression with the cholinergic midbrain marker, NADPH. The hM4Di protein (or mCherry control) seemed to be trafficked well throughout the cell as clear labelling of the fibers could be seen. We counted the number of cells labelled with mCherry (DAB-brown), NADPH (blue) and cells labelled with both. Overall, we estimated that the hM4Di-mCherry protein was expressed in 89% of PPT cholinergic neurons (mCherry control=87%). Representative images are shown in figure 4.15C and D. Of note there was a small percentage of neurons that were only mCherry positive (%mCherry cells that were cholinergic: hM4Di=88%, mCherry=90%), roughly half of these mCherry only cells were very small with a diameter of less than 10 µm.

All our behavioural and electrophysiological data was collected during the 21-35 day time-point and so we included only animals sacrificed at these time points for co-expression analysis as this more accurately represented viral expression during our experiments (mCherry n=4, hM4Di n=5). Animals sacrificed past this date had very strong expression of mCherry, with very heavy expression in areas the cholinergic PPT neurons project to (supplemental figure A.3). Using a fluorescent mCherry antibody, it was clear that this was due mCherry being expressed heavily by the axons and terminals of infected neurons. This occurred with both virus types. It may also be due to accumulated fluorescent toxicity, as has been suggested previously (Liu et al., 1999; for review see Allen et al., 2015).

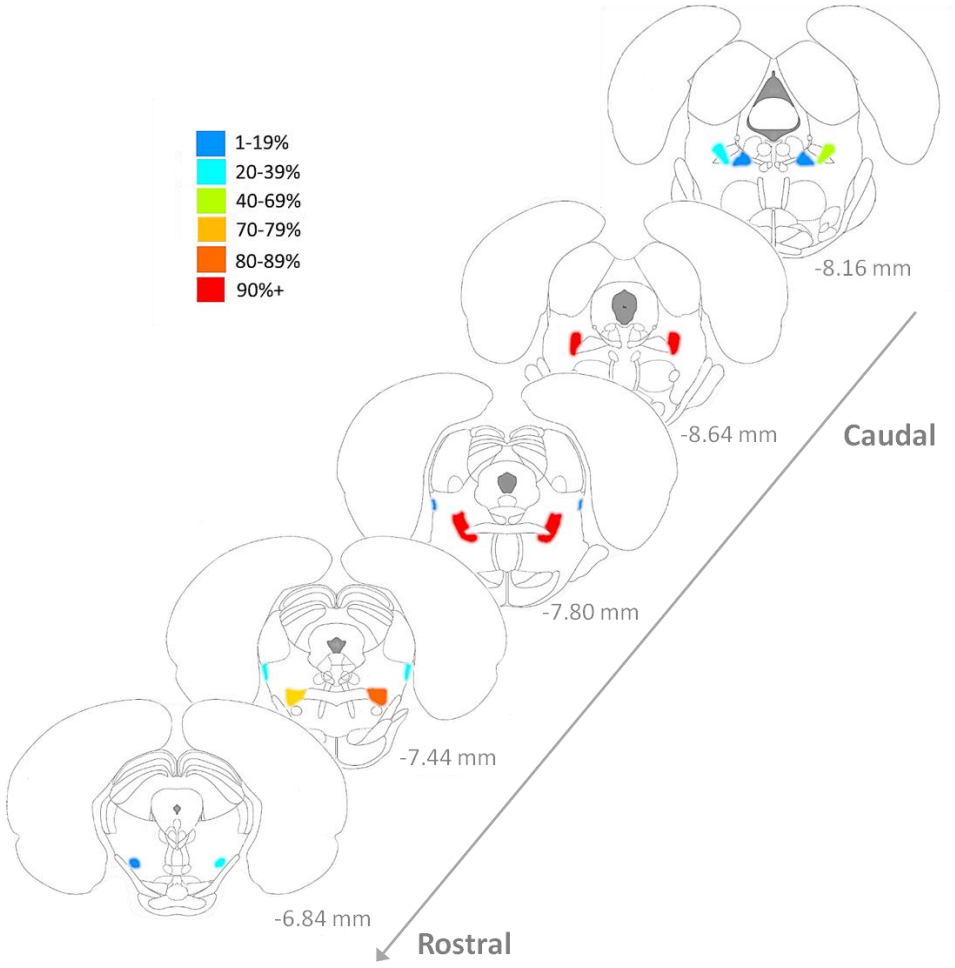

Figure 4.14 Expression of the hM4Di-mCherry Protein Throughout the PPT

To determine the efficacy of our viral injection we examined the expression of the viral tag mCherry in hM4Di expressing animals and plotted this across representative images of the PPT. We determined if mCherry expression within the PPT for each representative slice was present for each animal. Then the proportion of animals expressing mCherry at that representative slice was calculated (n=12). The highest proportion of animals (90%+) expressed mCherry in the caudal aspect of the PPT, and the lowest in the anterior (>20%). Some animals displayed mCherry expression in non-targeted regions, including the LDT (caudal aspect, shown in slice -8.16) and PGB (anterior aspect, -7.4-6.84 mm). The expression of control animals did not differ substantially (data not shown). Images were adapted from (Paxinos and Watson, 2005).

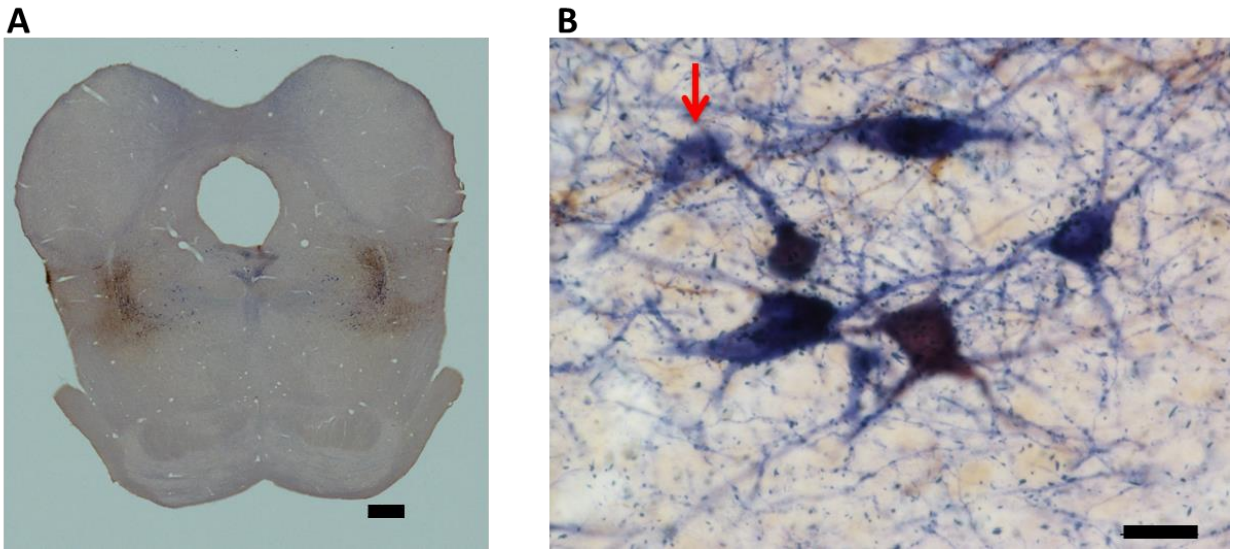

Figure 4.15 The hM4Di-tag mCherry is Co-expressed with the Cholinergic Marker, NADPH

In both images mCherry was labelled in brown (DAB), whereas NADPH (blue) marked midbrain cholinergic neurons. A) Representative image of mCherry and NADPH expression in the PPT (2x magnification). The scale bar represents 500 µm. B) Representative image of co-expression of NADPH and hM4Di-mCherry in the PPT at 20x magnification. Dually-labelled neurons appeared very dark blue, almost black, compared to cholinergic neurons not expressing mCherry (indicated by the red arrow). Overall, we determined that hM4Di-mCherry was expressed in 89% of cholinergic neurons. Here, the scale bar represents 25 µm.

4.3.2.9 Summary of Inhibition of the Cholinergic Neurons of the PPT

Overall, we saw that DREADD-induced inhibition of cholinergic cells using CNO improved short-term habituation of the ASR, and reduced baseline startle magnitude. We did not observe an impact on PPI or habituation of non-reflexive behaviours. Our control experiments show that DREADD-induced inhibition was effective at a cellular and population level as demonstrated by *in vitro* patch clamp recordings and *in vivo* electrophysiology. Furthermore we observed that multi-unit auditory responsiveness in the PPT was unaltered by DREADD-induced inhibition, complementing our finding of no impairment of auditory PPI. Finally, we confirmed that our DREADD-induced inhibition was able to alter behaviour as inhibiting cholinergic neurons of the PPT blocked morphine-induced CPP.

4.3.3 Activation of Cholinergic PPT Neurons Using Optogenetics

For this experiment we injected a Cre-dependant optogenetic virus (rAAV5-EF1α-DIO-ChR2(H134R)-EYFP, n=7, 1 male, 6 female), or the respective control virus (rAAV5-EF1α-DIO-EYFP, n=6, 1 male, 5 female) into the PPT and implanted a light fiber. Cells expressing ChR2(H134R) were activated by blue light. In this section, sex was not included as a factor in our statistical analysis because of the low number of males (n=1/group).

4.3.3.1 Startle Reactivity of ChR2 Expressing Animals

Prior to sensorimotor gating testing, all animals completed an I/O function, which assessed startle reactivity with increasingly intense auditory stimuli. During testing animals were tethered to the LED commutator, but received no light stimulation. As revealed by a two way repeated measures ANOVA (virus type × sound intensity) on unamplified startle amplitudes, both YFP only expressing controls and ChR2 animals show increasing startle magnitudes concordantly

with increasing sound intensity. We found a significant effect of sound level ($F_{(9,90)}$=9.2, p<0.001), but no effect of virus type ($F_{(1,10)}$=0.9, p=0.37) or interaction between virus and sound level ($F_{(9,90)}$=0.4, p=0.91). This indicates normal startle reactivity in both groups, without optogenetic stimulation, as shown in figure 4.16.

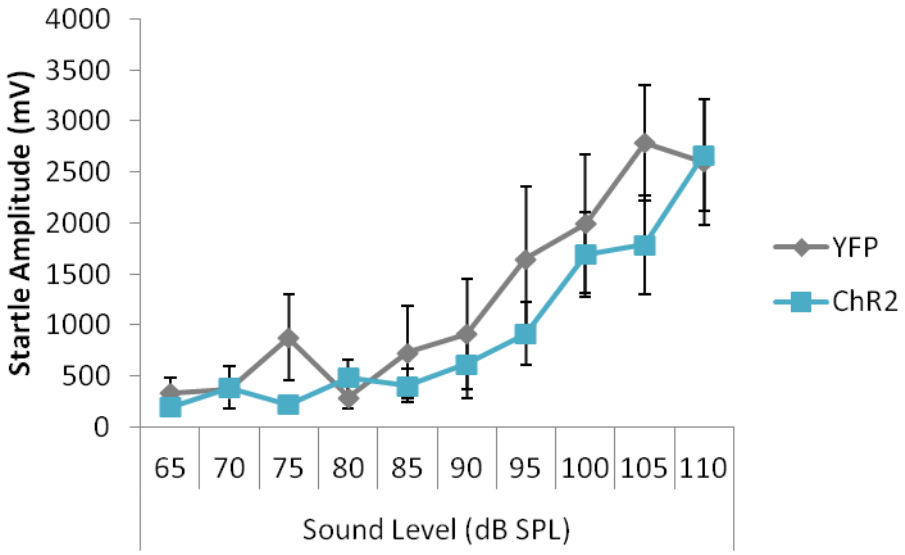

Figure 4.16 Startle Reactivity is Normal in ChR2 Expressing Animals

Both YFP controls and ChR2 expressing animals showed increasing startle responses with increasing sound intensity. This suggests that the expression of ChR2 - in contrast to DREADD expression - does not alter startle reactivity (YFP n=6, ChR2 n=7).

4.3.3.2 Activation of Cholinergic PPT Neurons and its Effects on Startle Magnitude

To see the effect of cholinergic PPT neuron activation on startle magnitude, we photostimulated these neurons simultaneously with the presentation of an auditory startle pulse. We individually normalized startle amplitudes by calculating a startle magnitude ratio (ratio: average startle magnitude with photostimulation/average startle magnitude without photostimulation). Differences from 1 indicated that photostimulation altered startle magnitude. As displayed in figure 4.17A, the control animals showed no change in startle magnitude with photostimulation as they had a ratio of 1.05 (±0.5), whereas ChR2 expressing animals had a ratio of 1.82 (±0.32), indicative of an increased startle magnitude with photostimulation. This difference in ratio was statistically significant (independent samples t-test: t_{11}=2.23, p=0.04). Interestingly this effect became greater as the number of consecutive trials with photostimulation progressed. This is shown in figure 4.17B. One animal was removed from ChR2 group as it was an outlier.

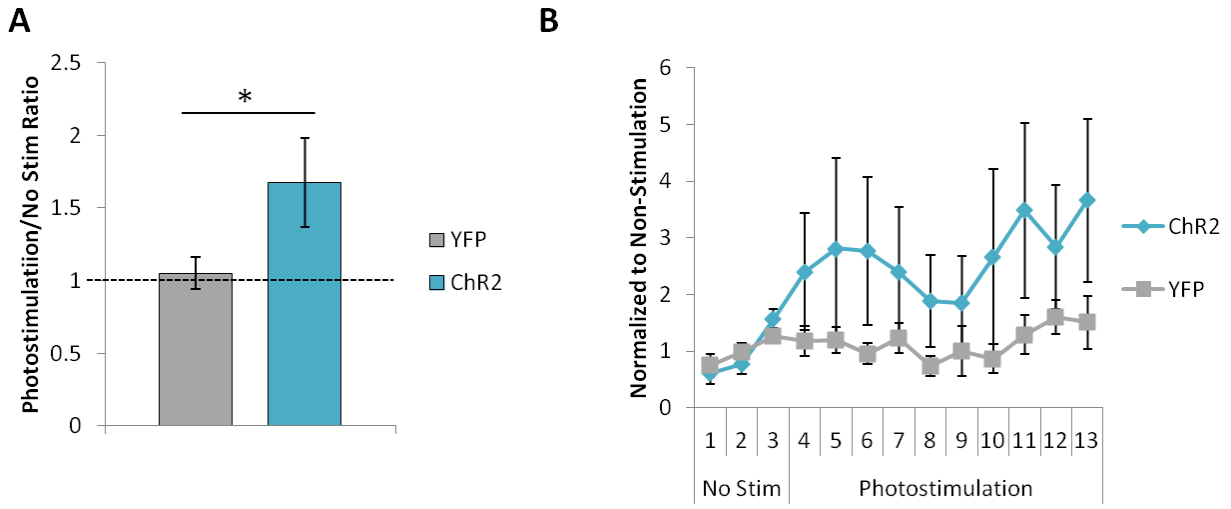

Figure 4.17 Photostimulation of Cholinergic PPT Neurons Increased Startle Magnitude

A) For each animal, we normalized startle amplitudes after photostimulation (Ratio: average startle amplitude with photostimulation/average without, 1=no change as indicated by the dotted line). ChR2 animals showed significantly increased startle magnitudes during stimulation compared to YFP controls. As shown in B) this increase appeared to be progressive. Startle magnitude was normalized to the first three trials prior to photostimulation for each animal.

4.3.3.3 Optogenetically-Induced PPI

If PPI is mediated by the activation of cholinergic PPT neurons inhibiting the startle pathway, we predicted we could induce PPI by activating these neurons prior to a startling sound. In order to investigate this, we administered several photostimulation frequencies and intensities, in both unilateral or bilateral stimulation paradigms, prior to the startle pulse (see appendix A for more detail). Within each testing session, we also presented trials with an auditory prepulse as a control condition (see figure 4.3).

Bilateral photostimulation of cholinergic PPT neurons at 50 Hz did not induce any inhibition of startle. In fact, similar to concurrent photostimulation, prior activation of cholinergic neurons facilitated startle and caused negative %PPI values, as shown in figure 4.18. We analysed this using a three way repeated measures ANOVA (type of prepulse × ISI × virus) and found a significant difference in %PPI depending on whether a optogenetic or auditory prepulse was administered ($F_{(1,11)}$=1106, p<0.001). Most importantly, we found a significant interaction between virus and prepulse type ($F_{(1,11)}$=97, p<0.001), which suggested that photostimulation influenced ChR2 animals and controls differently.

A follow-up ANOVA (virus × ISI) on %PPI using photostimulation prepulse conditions only, revealed that stimulation produced significantly different effects on ChR2 compared to YFP control animals across all ISIs ($F_{(1,5)}$=17, p<0.01). This effect was greatest at 15 ms, which induced an increase of startle magnitude by 86% (±13%) of baseline startle magnitude. Unilateral stimulation produced an increase in startle roughly half to bilateral stimulation (supplemental figure A.4), indicating that there is a dose effect depending on the amount of photostimulation administered before the startle stimulus.

As a control, within each testing session, auditory PPI was also assessed. Auditory prepulses (85 dB SPL) induced normal PPI in both ChR2 and control animals. We analyzed this using a follow up two way repeated measures ANOVA (virus × ISI) which showed no effect of virus type on PPI ($F_{(1,11)}$=0.4, p=0.54). In control animals PPI ranged from 67-85% depending on the ISI; similarly in ChR2 animals it ranged from 69-87% (figure 4.18), indicating that the lack of optogenetically-induced PPI was not due to a general PPI deficit in these animals.

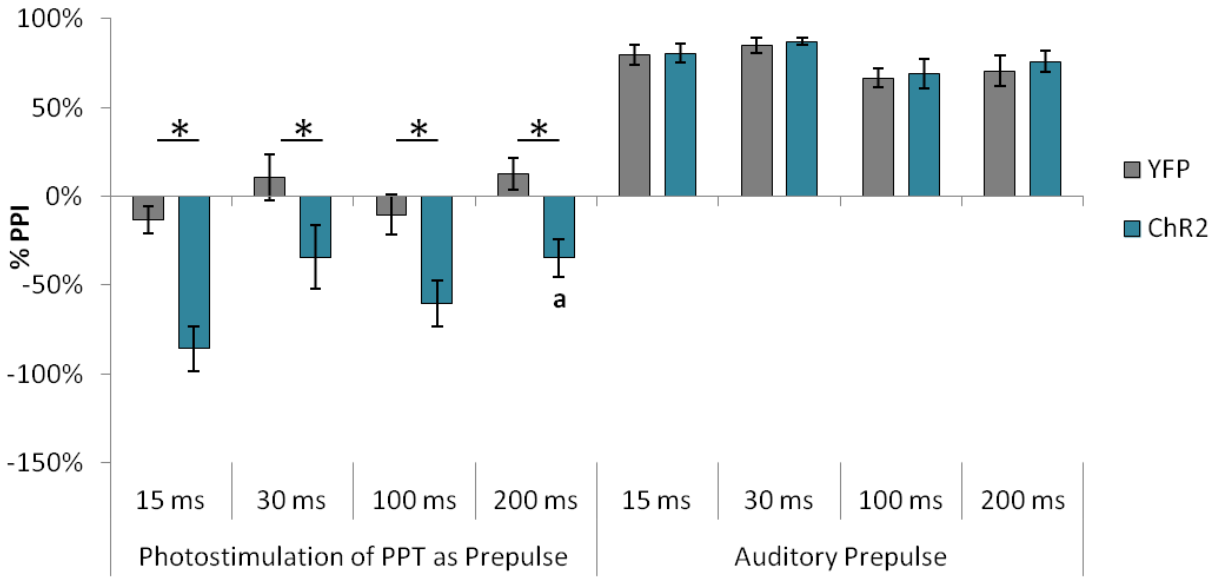

Figure 4.18 Activation of Cholinergic PPT Neurons as a Prepulse Induces Startle Facilitation

Bilateral activation of cholinergic PPT neurons induced a facilitation of startle magnitude in ChR2 expressing animals, which resulted in negative %PPI values (denoted startle amplitude with a prepulse relative to startle amplitude without). This effect was not present in YFP expressing control animals. The greatest facilitation of startle was observed at the 15 ms ISI (as denoted by *a*, which references that %PPI was statistically different at 200 vs 15 ms). Auditory prepulses (85 dB SPL) produced normal %PPI values in both ChR2 and YFP expressing animals, this suggests that our failure to optogenetically-induce PPI was not due to a general PPI impairment in these animals (ChR2 n=7, YFP n=6).

4.3.3.4 Optogenetically-Induced Startle Facilitation was Blocked by Systemic Nicotinic Antagonism

In order to gain some insight into what kind of ACh receptor subtype mediates the increase in startle by photostimulation, we examined if blockade of nicotinic receptors could prevent this increase across both photostimulation paradigms (photostimulation as a prepulse OR simultaneous photostimulation during a startling sound). Animals were injected with saline or the nAChR antagonist mecamylamine (3 mg/kg IP) one week apart, before they were re-tested with 50 Hz photostimulation.

We analyzed startle magnitude following photostimulation of the PPT as a prepulse using a three way repeated measures ANOVA (drug × virus × ISI). We reconfirmed that ChR2 expressing animals showed significantly increased startle during photostimulation as there was a main effect of virus type ($F_{(1,11)}$=13.5, p<0.01). We also found a significant difference between mecamylamine treatment and saline ($F_{(1,11)}$=15.6, p<0.01), and a significant interaction between drug and virus type ($F_{(1,11)}$=13.8, p<0.01). Post hoc t-tests revealed that, in ChR2 expressing animals, startle magnitude (measured by -%PPI values) significantly differed when give mecamylamine compared to saline (t_6=4.4, p<0.01); whereas in YFP controls this was not the case (t_5=1.0, p=0.18). Again, this effect was most prominent at the 15 ms ISI, where startle magnitude was facilitated by 37% by photostimulation under saline treatment (%PPI: -37 ±7%), but photostimulation had negligible effects under mecamylamine treatment as startle was magnitude was similar to baseline values without a prepulse (%PPI: 6 ±10%). Overall, in ChR2 animals, mecamylamine blocked the increase in startle magnitude by photostimulation across

ISIs as there was no interaction between drug and interstimulus interval ($F_{(3,33)}$=0.8, p=0.5; figure 4.19A).

As a control, we examined if baseline startle magnitude or auditory PPI was impacted by mecamylamine treatment. Basal startle amplitude (prior to photostimulation) was not different in ChR2 expressing animals when given saline or mecamylamine (t_6=1.7, p=0.14). With saline, raw startle values were 1335 mV (±240) and with nicotinic antagonism it was 1626 mV (±265). Additionally, auditory PPI was also unaffected by mecamylamine treatment (figure 4.19B). In a separate three way ANOVA (virus × drug × ISI) that examined auditory PPI, we found no effect of drug ($F_{(1,11)}$=1.0, p=0.34), or virus type ($F_{(1,11)}$=1.5, p=0.25) or interaction between these variables ($F_{(3,33)}$=0.1, p=0.96). This demonstrated that mecamylamine's actions were specific to blocking the startle facilitating effect via photostimulation of cholinergic PPT neurons.

Similar results were obtained using concurrent photostimulation of cholinergic PPT neurons with an acoustic startle sound (figure 4.19C). We analyzed individually normalized startle amplitudes (Ratio: photostimulation startle amplitude/startle without) using a two way repeated measures ANOVA (virus × drug). Again, we re-confirmed that 50 Hz stimulation caused a significant enhancement of startle magnitude in ChR2 animals, but not controls, as there was a significant effect of virus ($F_{(1,11)}$=6.9, p=0.02). The amount of enhancement with saline treatment, 1.70 (±0.31), was almost identical to that previously observed with our 50 Hz testing, 1.80 (±0.32), which demonstrated the stability of this effect (compare see figure 4.19C with 4.18A). Most importantly however, we found that there was a significant interaction between drug and virus type ($F_{(1,11)}$=6.2, p=0.03). Post-hoc tests revealed that mecamylamine treatment completely

blocked the optogenetically induced increase in startle in the ChR2 animals (t_6=2.5, p=0.04), whereas there was no effect of drug in YFP controls (t_6=1.0, p=0.36).

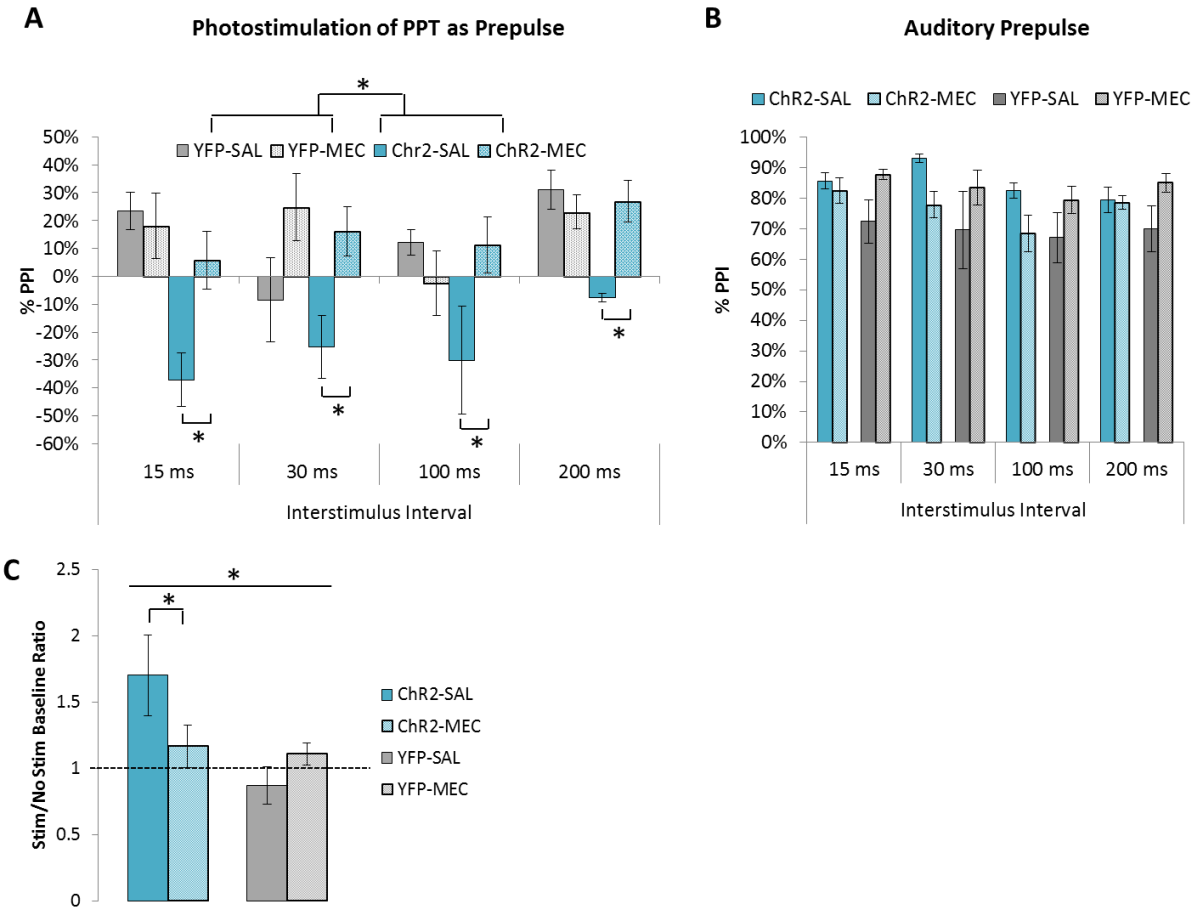

Figure 4.19 Nicotinic Antagonism Blocked Optogenetically-Induced Startle Facilitation

A) ChR2 and YFP control animals received an IP injection of saline (SAL) or mecamylamine (MEC), before undergoing photostimulation of cholinergic PPT neurons prior to a startling sound. Positive %PPI indicates startle inhibition, whereas negative values indicate startle facilitation. Photostimulation enhanced startle magnitude relative to when no prepulse was present in ChR2 animals. This was blocked by administration of mecamylamine. B) Presentation of an auditory prepulse prior to a startling pulse induced robust PPI in all animals, which was unaffected by

mecamylamine treatment in all groups. C) Mecamylamine was also able to prevent startle facilitation by concurrent photostimulation of cholinergic PPT neurons in ChR2 animals (YFP n=6, ChR2 n=7).

4.3.3.5 Optogenetically-Induced Conditioned Place Preference (Positive Behavioural Control)

It has been demonstrated previously that pairing optogenetic PPT stimulation with a context induces conditioned place preference (Xiao et al., 2016). To ensure that our photostimulation paradigm was effective at activating cholinergic PPT neurons we optogenetically induced CPP in ChR2 expressing animals as a positive behavioural control. Animals were repeatedly exposed to two different environments; one was paired with bilateral photostimulation of the PPT and the other was not. One animal from the ChR2 group was eliminated from analysis as it was unable to complete testing.

On test day, animals freely roamed the test chamber and the time spent in each environment type was recorded. We analysed this using a two way repeated measures ANOVA (environment × virus). Animals expressing ChR2 tended to spend more time in the photostimulation-paired environment (287 ± 26 s) compared to no stimulation-paired (215 ± 20s, see figure 4.20A). Control YFP animals spent a similar amount of time in the stimulation-paired environment as the unpaired (198 ± 24s and 238 ± 24s, respectively). However this trend just failed to reach statistical significance as we saw no effect of virus ($F_{(1,10)}$=4.3, p=0.06), environment ($F_{(1,10)}$=0.14, p=0.71) or interaction between these factors ($F_{(1,10)}$=2.3, p=0.16).

An individual preference score was calculated for each animal (time spent in paired/time spent in unpaired, 1=no preference). On average ChR2 animals had a preference score of 1.42

(±0.2), which was significantly different from 1 (one-tailed, one sample t-test: t_5=2.3, p=0.03). Control YFP animals had a preference score of 0.94 (±0.2), which was not significantly different from 1 (t_5=0.2, p=0.42; figure 4.20B). This suggests that photostimulation-induced a mild conditioned place preference in ChR2 animals but not controls.

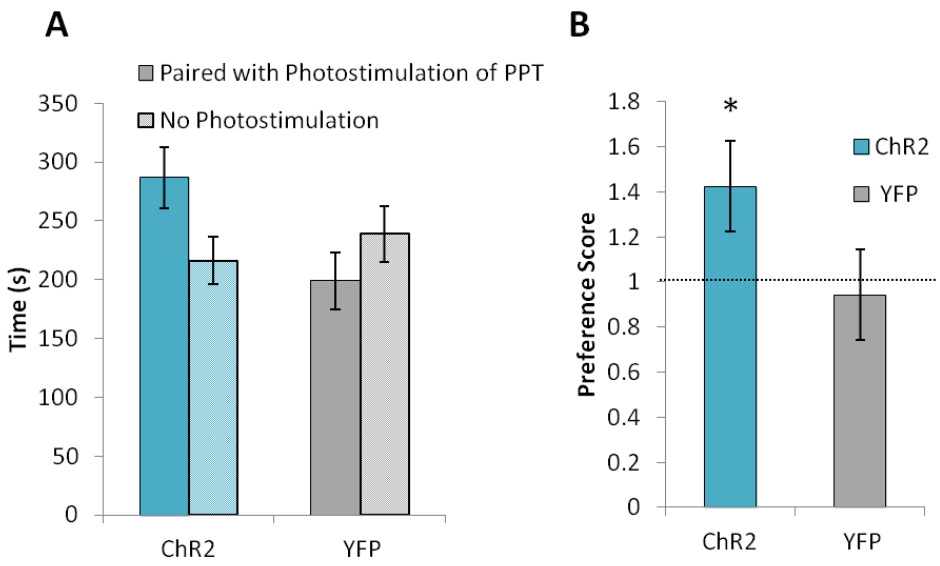

Figure 4.20 Activation of Cholinergic PPT Cells is Sufficient to Induce Mild CPP

Animals were exposed to two different environments, one paired with photostimulation of the PPT and one without. A) On test day, ChR2 animals tended to spend more time in the side paired with activation of cholinergic PPT neurons, however, this just failed to reach statistical significance (p=0.06). B) A preference score was calculated for each individual animal (time spent in stimulation-paired environment/time in non-stimulated environment, 1=no preference as indicated by the dotted line). ChR2 displayed a significant preference for the paired side, whereas YFP controls did not (n=6/group).

4.3.3.6 Verification of ChR2 Expression and Photostimulation Efficacy using c-FOS

All of the animals included in this analysis had bilateral expression of the either the virus containing the ChR2(H134R) opsin or YFP control. Placements of the light fiber implants were also verified to be within (or just above) the PPT (see figure 4.21). Although there was some spread in terms of the location of the light fiber, in general, most targeted the mid PPT.

We examined the expression of the viral-tag YFP and the co-expression with the cholinergic marker ChT or the cellular activation marker, c-FOS. The ChR2(H134R) protein (or YFP control) seemed to be trafficked through the cell well as clear labelling of many fibers could be seen. We counted the number of cells labelled with YFP, ChT (red) and cells labelled with both. Overall we estimated that the ChR2(H134R)-YFP protein was expressed in 70% of PPT cholinergic neurons (YFP control=73%). Inversely, 95% of YFP expressing neurons were cholinergic (ChR2(H134R), YFP controls: 89%). An example image is shown in figure 4.22A. This confirmed that ChR2 was expressed in the majority of PPT cholinergic neurons, but not in non-cholinergic neurons.

We then analysed the co-expression of YPF with the cellular activation marker, c-FOS. We found that our stimulation parameters induced c-FOS expression in 71% of ChR2(H134R)-YFP expressing neurons, whereas in YFP controls only 8% of YFP expressing cells also expressed c-FOS. An example image is shown in figure 4.22B. In both YFP control and ChR2(H134R) animals non-YFP labelled cells were also expressing c-FOS. This could be due to basal levels of expression, as animals were awake and mobile in their home-cages during photostimulation. We cannot rule out however, that the heat or light activated off-target cells. Overall however, our staining

indicated a good expression level of the optogenetic virus specifically in cholinergic cells, and that our photostimulation paradigm was sufficiently activating these cells.

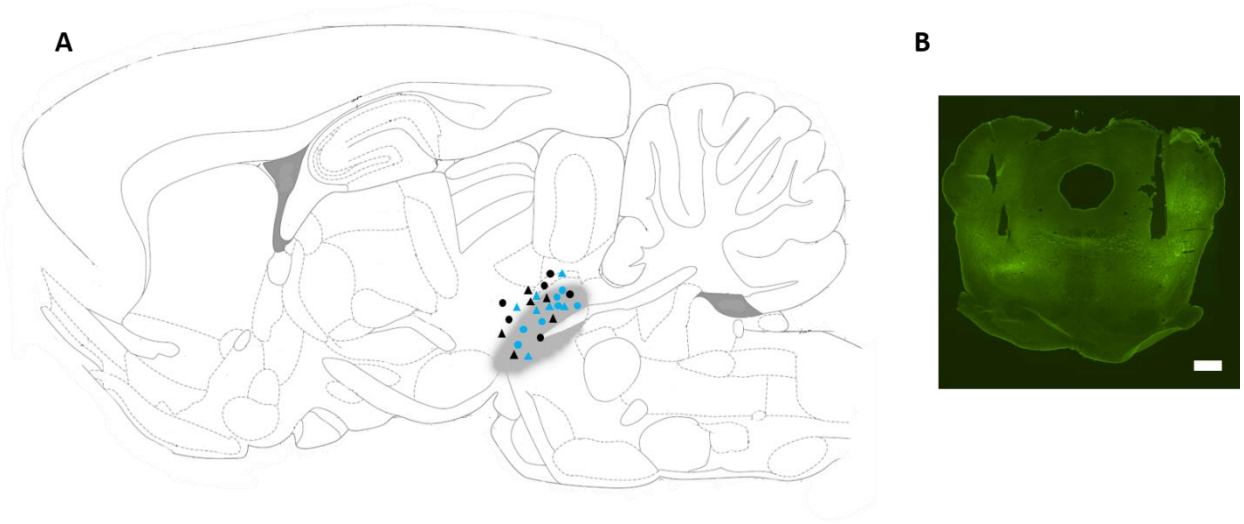

Figure 4.21 Placement of Bilaterally Implanted Light Fibers

A) This image displays the sagittal view of the PPT (medial/lateral plane: ±2.1). Shown in blue are the placements of the light fiber tips in ChR2(H134R) expressing animals, and black represents YFP controls. Triangles denote placement on the right side and dots on the left. It appeared that in both groups, the hits varied throughout the PPT, denoting good placement and stimulation. Images were adapted from (Paxinos and Watson, 2005). B) Is a representative image of the tracts of implanted light fibers. Scale bar is 500 µm.

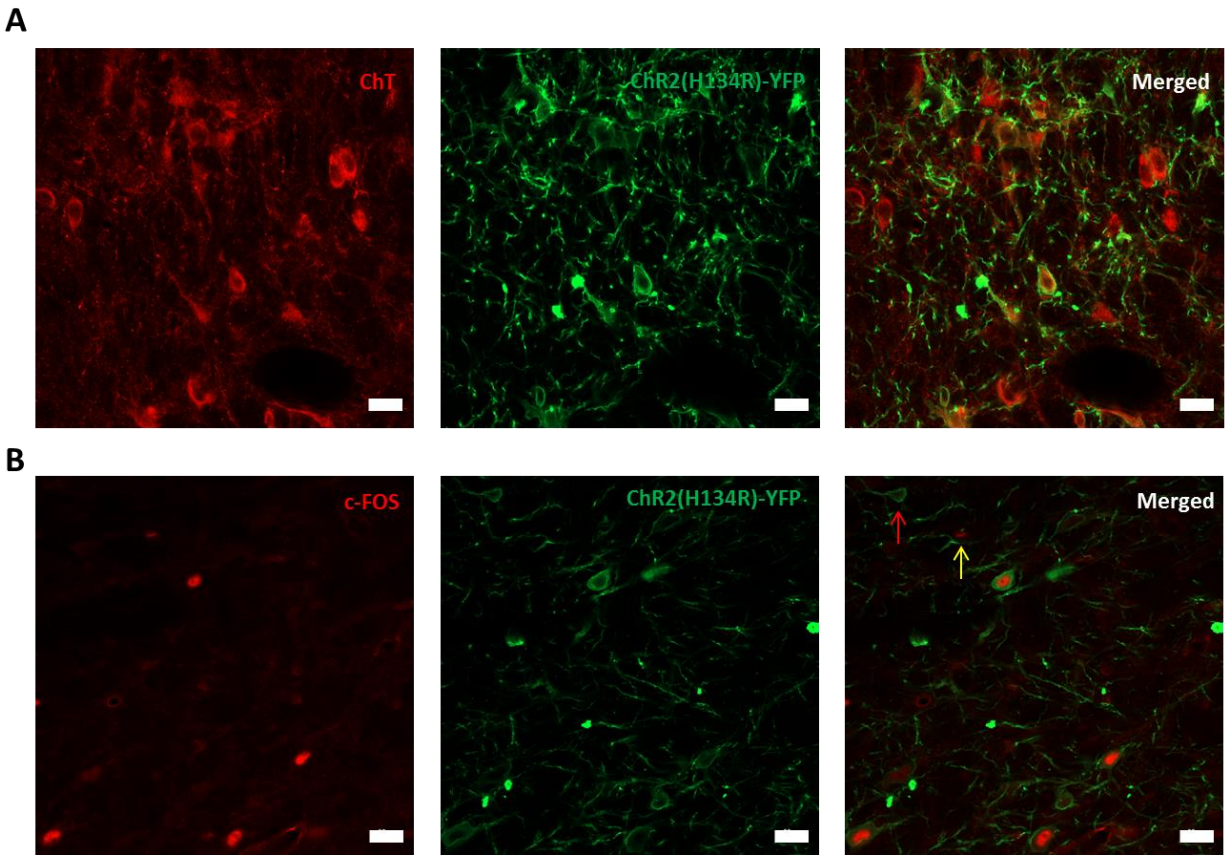

Figure 4.22 ChR2(H134R) is Expressed in Cholinergic Neurons and Photostimulation is Effective
A) Expression of ChR2(H134R and its co-expression with a cholinergic marker, ChT. Overall we estimated that the virus was expressed in 70% of cholinergic neurons within the PPT (YFP controls: 73%). Furthermore, we verified our photostimulation was effective using the cellular activation marker, c-FOS, shown in panel shown in panel B). Expression of c-FOS and ChR2(H134R)-YFP neurons: 71% of ChR2(H134R)-YFP neurons expressed c-FOS, (YFP control animals: 8%)

4.3.3.7 Summary of Cholinergic PPT Activation

Overall, we observed that activation of cholinergic PPT neurons facilitated startle. This was reliably observed if activation was during a startling sound or if activation was used as a prepulse. This enhancement was blocked by the nicotinic antagonist mecamylamine. We validated our optogenetic manipulations by recapitulating the previously reported induction of CPP through PPT cholinergic activation, as well as increased c-FOS activation in infected cholinergic neurons.

4.4 Discussion

The goal of these studies was to decipher the role of PPT cholinergic neurons in startle and startle modulation. The anatomical and cell-type specificity necessary for these experiments required the use of a transgenic rat model. As previously discussed, it is important to ensure that the transgene expression does not alter basal sensory filtering or sensorimotor gating abilities. As a first step, we therefore tested transgenic animals in comparison with WT littermates. We found that transgenic males had slightly lower maximal startle amplitude than their WT counterparts (figure 4.4B). The transgenic male's startle magnitude was still very robust, detectable, and identical to that of WT females. Additionally, all transgenic animals had the same startle threshold as WT (~85-90 dB SPL, figure 4.4A). This difference in maximum startle amplitude was not due to an effect of weight on signal detection as males from both genotypes weighed approximately the same. Regardless of this difference in startle reactivity, I found no differences in habituation (figure 4.6) or PPI (figure 4.7) of the ASR in transgenic rats. Moreover, we found no differences in the habituation of motivated behaviours or general locomotor

behaviour (figure 4.6). Therefore, I concluded that these animals were an appropriate model to study sensory filtering and sensorimotor gating mechanisms using optogenetics and DREADDS.

This study used two complimentary approaches to investigate cholinergic PPT contributions to startle, PPI and locomotor behaviour: neuronal silencing by DREADDS and optogenetic activation. We validated that methods were functional using electrophysiological recordings (figure 4.11 & 4.13) or c-FOS labelling (figure 4.22). Functionality at a cellular level, however, does not necessarily equate that a system will be sufficient to impact behaviour. Therefore, we included CPP a positive behavioural control. Inhibition of these neurons disrupted morphine-induced CPP (figure 4.13), whereas activation of these neurons were sufficient to induce CPP (Xiao et al., 2016, also see Gut & Winn, 2016 for discussion). We confirmed these findings (figures 4.13 and 4.20); as these served as a positive control measure, they will not be discussed further. Together, the control experiments across different levels of analysis suggest that both the DREADD inhibition and optogenetic activation were functional.

4.4.1 Prepulse Inhibition

According to the current hypothesis of the majority of sensorimotor gating literature, the major mechanism of PPI is an inhibition provided by cholinergic neurons of the PPT to startle-mediating areas of the brainstem, the PnC. To test this directly, we inhibited these neurons during sensorimotor gating testing using DREADDs. We found no impairment of PPI (figure 4.10A). Instead, we found that in hM4Di-expressing animals, CNO induced a general decrease in startle magnitude (figure 4.10B). The lack of PPI impairment following cholinergic PPT inhibition was not due to a dysfunctional DREADD system. We confirmed our cholinergic PPT inhibition was

functional through *in vitro* recordings at the neuronal level and observed robust inhibition (figure 4.11). Additionally when we performed *in vivo* electrophysiological recordings we saw a slight reduction in the spontaneous activity of multi-unit clusters following CNO administration (figure 4.12C & D). This was fitting with select inhibition of a subset of neurons within the PPT. We also examined auditory-evoked activity and found that cholinergic inhibition did not significantly alter auditory responsiveness within the PPT (figure 4.12E & F). If these neurons were critical for auditory PPI, we should have observed a decrease in auditory responsiveness.

Intact auditory processing and PPI post CNO administration provided a cohesive rationale for our findings and past studies (e.g. MacLaren et al., 2014), however it must be acknowledged that we inhibited these neurons, not irreversibly silenced them. Activation of the hM4Di protein hyperpolarizes neurons via activation of potassium channels (Armbruster et al., 2007) and inhibits presynaptic neurotransmitter release (Stachniak et al., 2014). It cannot be ruled out that suprathreshold stimuli may have overpowered this inhibition. However, our complimentary optogenetic data suggests this was not a large factor. In general, our findings mirror the effects of a cholinergic lesion by a diphtheria toxin-fusion protein as reported by MacLaren et al. (2014), however, their reduction in startle magnitude was much more profound. Our hM4Di tag, mCherry, was highly expressed by cholinergic neurons and expression spread throughout the majority of the PPT (figures 4.15 and 4.16), much like the spread and efficacy of the previously reported lesions. Therefore, differences in the magnitude of startle reduction between these two studies may highlight the difference between temporary inhibition compared to total removal of these neurons.

To further address the role of cholinergic PPT neurons in PPI, we also attempted to induce PPI by optogenetically activating these neurons prior to a startling sound – basically to see if photostimulation could replace the acoustic prepulse. Instead of inhibiting startle, we found activation of these neurons enhanced startle, resulting in negative PPI values, or startle facilitation. The lack of optogenetically-induced PPI was not due to an impairment of PPI in general as auditory prepulses reliably induced PPI (figure 4.18). Moreover, when we photostimulated these neurons during a startle response, this also increased startle magnitude (figure 4.17A). This nicely compliments our hM4Di-induced reduction in startle, indicating that cholinergic neurons in the PPT can indeed modulate startle responses, but they seem to facilitate startle rather than inhibit it, ruling out any major role in the mediation of PPI.

Our findings add to the growing body of more recent literature that has started to suggest that PPT cholinergic neurons are not the primary mechanism underlying PPI. While this contradicts the majority of the traditional predictions within the field, there is now converging evidence using both acute (current study) and chronic manipulations (Machold, 2013; MacLaren et al., 2014) of spatially restricted cholinergic function. Therefore, we suggest that ACh modulates PPI, presumably at the level of the PPT, but it does not mediate PPI. It is likely that other cells, within the PPT and/or even outside of it, play a more important role than previously assumed. Future studies should seek to re-examine the neural circuitry of PPI and test if GABAergic and/or glutamatergic PPT neurons are responsible for PPI.

4.4.2 Modulation of Startle Magnitude

Both of our complimentary methods found that cholinergic PPT neurons modulated startle reactivity: inhibition reduced startle magnitude whereas activation increased it. Thus, the role of cholinergic midbrain neurons seems to be to maintain or enhance startle reactivity. This is fitting with the cholinergic PPT neuron's role in arousal. These neurons have been demonstrated to be critical in sleep-wake transitions (Van Dort et al., 2015), and are part of the Ascending Reticular Activating System. Increased startle reactivity may be a reflection of increased arousal. Overall, based on our findings, we predict that the PPT acts as an integration point to modulate startle magnitude.

Stimulation of cholinergic PPT neurons enhanced startle magnitude (figure 4.17B & figure 4.18A), fitting with sensitization and/or PPF. Our observed improvement of short-term habituation of the ASR was surprising when examined in within the scope of previous literature as ACh has not been linked short-term habituation of the ASR. However, our results would be very logical if these neurons are involved in sensitization of startle. According to the dual process theory, behavioural output is the sum of the opposing forces: habituation and sensitization (Groves and Thompson, 1970). Inhibition of these neurons could have inhibited sensitization, resulting in improved short-term habituation. Indeed, our observed enhancement of short-term habituation had the greatest influence on the rate of habituation, i.e. at early trials, which are supposedly influenced by sensitization to a greater degree than later trials, where sensitization subsides (Pilz and Schnitzler, 1996). Additionally, cholinergic drugs have been known to generally enhance startle magnitude (see Chapter 3 or Acri et al., 1995; Philippens et al., 1997), but little research has investigated if these types of effects contribute to the mechanism of startle

sensitization. Furthermore, as these studies have used systemic manipulations it is unclear to what extent cholinergic effects on startle magnitude are due to modulatory brain regions, or due to side effects on motor systems. Our evidence highlights a potential central mechanism of sensitization that involves the cholinergic cells of the PPT.

It has been well documented that substance P is involved in the sensitization of the ASR. Local infusions of substance P into the PnC increased startle magnitude; furthermore, antagonism of this neuropeptide prevented sensitization of startle via foot shocks (Krase et al., 1993). Interestingly, Kungel et al. (1994) found that the substance P innervation of the PnC mainly came from the PPT and LDT. They also observed that substance P's ability to increase excitability of PnC neurons was increased with the co-administration of a cholinergic agonist. An estimated 30% of cholinergic neurons in the PPT co-express substance P markers (Standaert et al., 1986). At this point, it is unclear if these neurons modulate startle in the same manner as solely cholinergic neurons or not. It could be that cholinergic neurons that co-express substance P have a role in sensitization and enhance startle magnitude, whereas those that only release ACh are important for PPI and inhibit startle magnitude. Both our DREADD and optogenetic methods would have infected both types of cholinergic neurons. If this was the case, it appears that cholinergic substance P expressing neurons have a dominant effect on startle magnitude that overshadowed any inhibition of startle. This would be fitting with the well-documented inhibition of giant PnC neurons that occurs *in vitro* (Bosch and Schmid, 2006, 2008; Pinnock et al., 2015).

However, there is evidence to suggest that this dual role of cholinergic neurons is not feasible. Our findings of unimpaired auditory processing within the PPT following inhibition of cholinergic neurons indicate that non-cholinergic neurons are better suited to provide the

inhibition necessary for PPI. Additionally, i*n vitro* recordings of unidentified PnC neurons in the cat following electrical stimulation of the PPT caused prolonged excitatory responses which was blocked by administration of scopolamine, a muscarinic antagonist, and could be induced using an ACh agonist (Homma et al., 2002). Therefore, we propose that ACh may have differential effects depending on cell types within the PnC, and/or depending on whether it is synaptically released or tonically present. However, it remains undetermined how cells within the PnC incorporate various inputs to determine behavioural output, or how other cell types modulate giant-neuron activity. Regardless, the dominant effect of midbrain cholinergic regulation appeared to be an increase in PnC excitability, which resulted in higher startle amplitudes.

4.4.3 Nicotinic Receptors and Startle Magnitude

Our optogenetically-induced enhancement of startle magnitude was blocked by systemic administration of the nicotinic antagonist, mecamylamine (figure 4.19A). Although it robustly reduced the enhancement of startle, mecamylamine treatment did not alter auditory PPI (figure 4.19B) consistent with previous studies (Curzon et al., 1994; Higashino et al., 2016). Although here we have shown that nicotinic receptors are critical for optogenetically-induced enhancement, it is not clear which nAChR subtype is responsible. In general, systemic nicotine and nicotinic receptor agonism have been well documented to increase startle magnitude and improve PPI (Acri et al., 1994; Acri et al., 1995). In Chapter 3 we suggested that the α7-nAChR is critical for the systemic nicotine effect. However, local infusions of nicotine directly into the PnC have been shown to disrupt PPI, which appeared to be mediated by α4-β2 nAChRs and not α7-nAChRs. Therefore, both α4-β2 nAChRs as well as α7-nAChRs seem to modulate startle, although apparently at different brain regions. Whereas there is evidence that α4-β2 nAChRs are

expressed in the PnC, startle modulation by α7-nAChRs seems to be mediated by higher brain structures (Pinnock et al., 2015). Both could be involved in our observed nicotinic-mediated modulation of startle reactivity. Moreover, it is important to note that chronic activation of nicotine receptors by agonists might lead to receptor internalization, thereby rendering them antagonists, hence any study involving the application of nicotine or other agonists must be interpreted very carefully.

Local circuitry within the PnC and PPT is poorly understood. It is important to keep in mind that the optogenetic and chemogenetic manipulations of PPT cholinergic neurons could have impacted either inhibitory glycinergic or GABAergic interneurons or other excitatory cell types within the PPT or PnC, which ultimately promoted excitability of the giant-neurons and enhanced startle. Additionally, cholinergic PPT neurons also project to several other areas known to be critical for startle, including the inferior colliculus (Semba and Fibiger, 1992) and cochlear nucleus (Mellott et al., 2011). For example, it has been shown that cholinergic agonism within the cochlear nucleus increased neuronal activity (Chen et al., 1998), which could fit with our observed results. As we have stated before, studies have also shown that within the PnC substance P induces excitatory responses of unidentified neuron types, and that was enhanced by cholinergic agonism (Kungel et al., 1994). Therefore, it could be that some of our findings are also due to the actions of substance P.

Overall, we conclude that there is no simplistic circuit that could reconcile all observations with regards to cholinergic modulation of startle. But regardless of where the mechanism of action occurs, it is clear from our data that cholinergic projections from the PPT serve to modulate startle, more specifically enhance it, and that this is at least partially mediated though nicotinic

receptor activation. Future experiments need to address the details of neural circuitry and synaptic mechanisms.

4.4.4 Habituation of the ASR and Locomotor Behaviour

In contrast to effect on short-term habituation of the ASR, we did not observe any influence on the short-term habituation of the motivated behaviour, i.e. locomotion (figure 4.9C & D). Habituation of the ASR and locomotor behaviour have been long known to be differentially regulated (Hughes, 1984). Here we provide evidence that sensitization of these behaviours are also differentially regulated. Our evidence suggests that sensitization (or enhanced arousal, future studies will need to pinpoint the mechanism underlying our observed startle modulation) mechanisms do not reflect a global heightening of excitability that robustly enhances all behavioural responding. Instead, sensitization of different behaviours appeared to be differentially mediated.

ACh has been previously documented to be important for both the short- and long-term habituation of locomotor behaviour (Schildein et al., 2002; Lamprea et al., 2003; Dere et al., 2008). In particular, cholinergic activity within the nucleus accumbens, an area the PPT projects to (Dautan et al., 2014), has been particularly implicated (Schildein et al., 2002). However, our data indicates that the midbrain cholinergic neurons are not providing the input necessary for this behaviour. Other cholinergic centers may relay this information to the nucleus accumbens. Indeed, it has been shown that habituation of locomotor behaviour was more strongly correlated with cholinergic activity in the hippocampus (Thiel et al., 1998; Giovannini et al., 2001).

Finally, the PPT has been traditionally thought of as a nucleus that is highly important for motor control, specifically locomotion. In a recent review it was suggested that the PPT does not directly mediate locomotion, but can modulate it through its participation in part of a lower-level action selection process (Gut and Winn, 2016). We observed no impairment in locomotion following cholinergic inhibition of the PPT (figure 4.9D) and no impact of cholinergic activation on locomotor behaviour (data not shown). This is fitting with the newly conceptualized role of the PPT as part of a low level action selection circuit, as proposed by Gut and Winn (2016).

4.4.5 Caveats

Like any study, our observations should be considered with a few caveats in mind. Firstly, although we found no alterations in the sensory filtering or sensorimotor gating capabilities of within the transgenic Cre-ChAT rat line, transgenic animal models may not always best reflect normal physiological functioning. Transgenic animals can have off-target effects that alter behaviour, cognitive, or muscular function (e.g. see Kolisnyk et al., 2013). Although we found no major indication that this was the case, and others have published behavioural studies using this identical line (Pienaar et al., 2015; Xiao et al., 2016), there could be off-target effects that we are not currently aware of.

With regards to our DREADD-induced inhibition of the cholinergic PPT, we found some unexpected data that should be noted. Firstly, we saw that startle magnitude and reactivity was different between hM4Di-expressing animals and mCherry controls (figure 4.8). This was of special concern because this testing was conducted without administration of CNO. Cre-ChAT rats (with no virus injection) show a very similar curve to that of mCherry control Cre-ChAT rats

(compare with figure 4.4) indicating that the surgical procedure itself did not impact this. Therefore, this implies that the hM4Di receptor potentially had constitutive or basal activity that did not require ligand binding. A recent paper by Roth (2016) has reviewed how high expression levels of DREADD proteins may be particularly susceptible to this. As we observed very robust inhibition at the neuronal level (figure 4.11) and mCherry labelling was very dark and trafficked well throughout the entire neuronal body and processes (figure 4.15), it is likely that this accounted for our observed increase in startle reactivity. Furthermore, during our *in vivo* electrophysiological recordings it is possible that we again detected this constitutive activity: the signal to noise ratio we observed specifically in hM4Di infected PPT neuronal recordings prevented us from being able to isolate single-unit data. This was not a technical issue because we were able to decipher single-unit activity in mCherry controls. Why constitutive activity of an inhibitory receptor would increase startle reactivity or alter the noise-floor of electrophysiological recordings is unknown, but it might be through non-specific effects on any signalling pathway within the cell.

Lastly, it should be noted that although optogenetic stimulation is a powerful tool, the stimulation frequency and intensity used in this study likely does not accurately reflect endogenous physiological activity of cholinergic PPT neurons. This is a frequent critique of optogenetics as it is still a new technology (Boyden, 2015; Deisseroth, 2015). Our stimulation paradigm was 50 Hz, but the maximum frequency the ChR2(H134R) opsin can entrain to is likely around 40 Hz (Fenno et al., 2011). It is unknown which stimulation frequency would be best suited to capture the natural activity of PPT cholinergic neurons, if it is possible at all. Instead, we decided to use a stimulation paradigm that presumably induced maximum cholinergic-PPT

activation. Therefore, our high photostimulation frequency should induce robust, but not time-locked, neuronal activity. Future studies should attempt to modulate neuronal activity in a more physiologically relevant manner when possible.

4.4.6 Conclusions

This study has uncovered an exciting role for midbrain cholinergic neurons in the modulation of startle. Fitting with more recent studies by others, we were unable to find any alteration of PPI with cholinergic PPT manipulation. Our DREADD and optogenetic systems were validated at the neuronal and behavioural level which indicated that the lack of on impact on PPI was not due to methodological issues. This adds to the growing evidence suggesting that activation of these neurons are not the primary mechanism underlying PPI (see also Machold, 2013; MacLaren et al., 2014). Instead, we found that midbrain cholinergic function serves to generally modulate startle reactivity. Inhibition of these neurons decreases startle, whereas activation reliably enhances it, in accordance to the role of cholinergic PPT neurons in arousal and hinting on a potential role in startle sensitization. There is no impact of these neurons on locomotor behaviour. Although we have implicated nicotinic receptors in mediating this enhancement of startle magnitude, future studies will have to determine if other receptors play a role, and at what part in the startle circuitry this effect occurs. Although we did not discuss the role of the cholinergic midbrain in CPP here this is an exciting development. Future studies may also want to better examine the impact on VTA and nucleus accumbens dopaminergic signalling, and what receptors are responsible for this as well. Again, this may lead to a better understanding about how the cholinergic cells serve to encode context, and if this is related to their function in arousal.

4.5 References

Acri JB, Morse DE, Popke EJ, Grunberg NE (1994) Nicotine increases sensory gating measured as inhibition of the acoustic startle reflex in rats. Psychopharmacology (Berl) 114:369-374.

Acri JB, Brown KJ, Saah MI, Grunberg NE (1995) Strain and age differences in acoustic startle responses and effects of nicotine in rats. Pharmacol Biochem Behav 50:191-198.

Ahmad T, Lauzon NM, de Jaeger X, Laviolette SR (2013) Cannabinoid transmission in the prelimbic cortex bidirectionally controls opiate reward and aversion signaling through dissociable kappa versus mu-opiate receptor dependent mechanisms. J Neurosci 33:15642-15651.

Allen BD, Singer AC, Boyden ES (2015) Principles of designing interpretable optogenetic behavior experiments. Learn Mem 22:232-238.

Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc Natl Acad Sci U S A 104:5163-5168.

Bosch D, Schmid S (2006) Activation of muscarinic cholinergic receptors inhibits giant neurones in the caudal pontine reticular nucleus. Eur J Neurosci 24:1967-1975.

Bosch D, Schmid S (2008) Cholinergic mechanism underlying prepulse inhibition of the startle response in rats. Neuroscience 155:326-335.

Boyden ES (2015) Optogenetics and the future of neuroscience. Nat Neurosci 18:1200-1201.

Braff DL, Geyer MA (1990) Sensorimotor gating and schizophrenia. Human and animal model studies. Arch Gen Psychiatry 47:181-188.

Chen K, Waller HJ, Godfrey DA (1998) Effects of endogenous acetylcholine on spontaneous activity in rat dorsal cochlear nucleus slices. Brain Res 783:219-226.

Curzon P, Kim DJ, Decker MW (1994) Effect of nicotine, lobeline, and mecamylamine on sensory gating in the rat. Pharmacol Biochem Behav 49:877-882.

Dautan D, Huerta-Ocampo I, Witten IB, Deisseroth K, Bolam JP, Gerdjikov T, Mena-Segovia J (2014) A major external source of cholinergic innervation of the striatum and nucleus accumbens originates in the brainstem. J Neurosci 34:4509-4518.

Debruyne D, Sobrio F, Hinschberger A, Camsonne R, Coquerel A, Barre L (2003) Short-term pharmacokinetics and brain distribution of mecamylamine as a preliminary to carbon-11 labeling for nicotinic receptor investigation. J Pharm Sci 92:1051-1057.

Deisseroth K (2015) Optogenetics: 10 years of microbial opsins in neuroscience. Nat Neurosci 18:1213-1225.

Dere E, Zheng-Fischhofer Q, Viggiano D, Gironi Carnevale UA, Ruocco LA, Zlomuzica A, Schnichels M, Willecke K, Huston JP, Sadile AG (2008) Connexin31.1 deficiency in the mouse impairs object memory and modulates open-field exploration, acetylcholine esterase levels in the striatum, and cAMP response element-binding protein levels in the striatum and piriform cortex. Neuroscience 153:396-405.

Fendt M, Koch M (1999) Cholinergic modulation of the acoustic startle response in the caudal pontine reticular nucleus of the rat. Eur J Pharmacol 370:101-107.

Fenno L, Yizhar O, Deisseroth K (2011) The development and application of optogenetics. Annu Rev Neurosci 34:389-412.

Garcia-Rill E, Skinner RD, Miyazato H, Homma Y (2001) Pedunculopontine stimulation induces prolonged activation of pontine reticular neurons. Neuroscience 104:455-465.
Giovannini MG, Rakovska A, Benton RS, Pazzagli M, Bianchi L, Pepeu G (2001) Effects of novelty and habituation on acetylcholine, GABA, and glutamate release from the frontal cortex and hippocampus of freely moving rats. Neuroscience 106:43-53.
Graham FK (1975) Presidential Address, 1974. The more or less startling effects of weak prestimulation. Psychophysiology 12:238-248.
Groves PM, Thompson RF (1970) Habituation: a dual-process theory. Psychol Rev 77:419-450.
Guettier JM, Gautam D, Scarselli M, Ruiz de Azua I, Li JH, Rosemond E, Ma X, Gonzalez FJ, Armbruster BN, Lu H, Roth BL, Wess J (2009) A chemical-genetic approach to study G protein regulation of beta cell function in vivo. Proc Natl Acad Sci U S A 106:19197-19202.
Gut NK, Winn P (2016) The pedunculopontine tegmental nucleus-A functional hypothesis from the comparative literature. Mov Disord 31:615-624.
Higashino K, Ago Y, Umeki T, Hasebe S, Onaka Y, Hashimoto H, Takuma K, Matsuda T (2016) Rivastigmine improves isolation rearing-induced prepulse inhibition deficits via muscarinic acetylcholine receptors in mice. Psychopharmacology (Berl) 233:521-528.
Hoenig K, Hochrein A, Quednow BB, Maier W, Wagner M (2005) Impaired prepulse inhibition of acoustic startle in obsessive-compulsive disorder. Biol Psychiatry 57:1153-1158.
Hohnadel E, Bouchard K, Terry AV, Jr. (2007) Galantamine and donepezil attenuate pharmacologically induced deficits in prepulse inhibition in rats. Neuropharmacology 52:542-551.
Homma Y, Skinner RD, Garcia-Rill E (2002) Effects of pedunculopontine nucleus (PPN) stimulation on caudal pontine reticular formation (PnC) neurons in vitro. J Neurophysiol 87:3033-3047.
Hughes RN (1984) Cholinergic and monoaminergic substrates of startle habituation. Life Sci 34:2101-2105.
Ison JR, McAdam DW, Hammond GR (1973) Latency and amplitude changes in the acoustic startle reflex of the rat produced by variation in auditory prestimulation. Physiol Behav 10:1035-1039.
Ison JR, Taylor MK, Bowen GP, Schwarzkopf SB (1997) Facilitation and inhibition of the acoustic startle reflex in the rat after a momentary increase in background noise level. Behav Neurosci 111:1335-1352.
Koch M (1999) The neurobiology of startle. Prog Neurobiol 59:107-128.
Koch M, Kungel M, Herbert H (1993) Cholinergic neurons in the pedunculopontine tegmental nucleus are involved in the mediation of prepulse inhibition of the acoustic startle response in the rat. Exp Brain Res 97:71-82.
Kolisnyk B, Guzman MS, Raulic S, Fan J, Magalhaes AC, Feng G, Gros R, Prado VF, Prado MA (2013) ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. J Neurosci 33:10427-10438.
Krase W, Koch M, Schnitzler HU (1993) Glutamate antagonists in the reticular formation reduce the acoustic startle response. Neuroreport 4:13-16.

Krase W, Koch M, Schnitzler HU (1994) Substance P is involved in the sensitization of the acoustic startle response by footshocks in rats. Behav Brain Res 63:81-88.
Kungel M, Ebert U, Herbert H, Ostwald J (1994) Substance P and other putative transmitters modulate the activity of reticular pontine neurons: an electrophysiological and immunohistochemical study. Brain Res 643:29-39.
Lamprea MR, Cardenas FP, Setem J, Morato S (2008) Thigmotactic responses in an open-field. Braz J Med Biol Res 41:135-140.
Lamprea MR, Cardenas FP, Silveira R, Walsh TJ, Morato S (2003) Effects of septal cholinergic lesion on rat exploratory behavior in an open-field. Braz J Med Biol Res 36:233-238.
Laviolette SR, van der Kooy D (2003) The motivational valence of nicotine in the rat ventral tegmental area is switched from rewarding to aversive following blockade of the alpha7-subunit-containing nicotinic acetylcholine receptor. Psychopharmacology (Berl) 166:306-313.
Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ (1999) Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 260:712-717.
Liu X, Ramirez S, Pang PT, Puryear CB, Govindarajan A, Deisseroth K, Tonegawa S (2012) Optogenetic stimulation of a hippocampal engram activates fear memory recall. Nature 484:381-385.
Machold RP (2013) Loss of rostral brainstem cholinergic activity results in decreased ultrasonic vocalization behavior and altered sensorimotor gating. Behav Brain Res 256:51-55.
MacLaren DA, Markovic T, Clark SD (2014) Assessment of sensorimotor gating following selective lesions of cholinergic pedunculopontine neurons. Eur J Neurosci 40:3526-3537.
Mahler SV, Vazey EM, Beckley JT, Keistler CR, McGlinchey EM, Kaufling J, Wilson SP, Deisseroth K, Woodward JJ, Aston-Jones G (2014) Designer receptors show role for ventral pallidum input to ventral tegmental area in cocaine seeking. Nat Neurosci 17:577-585.
Mellott JG, Motts SD, Schofield BR (2011) Multiple origins of cholinergic innervation of the cochlear nucleus. Neuroscience 180:138-147.
Ornitz EM, Lane SJ, Sugiyama T, de Traversay J (1993) Startle modulation studies in autism. J Autism Dev Disord 23:619-637.
Paxinos G, Watson C (2005) The rat brain in stereotaxic coordinates, 5th Edition Edition. Burlington, MA: Elsevier Academic Press.
Philippens IH, Olivier B, Melchers BP (1997) Effects of physostigmine on the startle in guinea pigs: two mechanisms involved. Pharmacol Biochem Behav 58:909-913.
Pienaar IS, Gartside SE, Sharma P, De Paola V, Gretenkord S, Withers D, Elson JL, Dexter DT (2015) Pharmacogenetic stimulation of cholinergic pedunculopontine neurons reverses motor deficits in a rat model of Parkinson's disease. Mol Neurodegener 10:47.
Pilz PK, Schnitzler HU (1996) Habituation and sensitization of the acoustic startle response in rats: amplitude, threshold, and latency measures. Neurobiol Learn Mem 66:67-79.
Pinnock F, Bosch D, Brown T, Simons N, Yeomans JR, DeOliveira C, Schmid S (2015) Nicotine receptors mediating sensorimotor gating and its enhancement by systemic nicotine. Front Behav Neurosci 9:30.

Pitchers KK, Frohmader KS, Vialou V, Mouzon E, Nestler EJ, Lehman MN, Coolen LM (2010) DeltaFosB in the nucleus accumbens is critical for reinforcing effects of sexual reward. Genes Brain Behav 9:831-840.
Roth BL (2016) DREADDs for Neuroscientists. Neuron 89:683-694.
Schildein S, Huston JP, Schwarting RK (2002) Open field habituation learning is improved by nicotine and attenuated by mecamylamine administered posttrial into the nucleus accumbens. Neurobiol Learn Mem 77:277-290.
Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676-682.
Schmid S, Azzopardi E, De Jaeger X, Prado MA, Prado VF (2011) VAChT knock-down mice show normal prepulse inhibition but disrupted long-term habituation. Genes Brain Behav 10:457-464.
Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671-675.
Semba K, Fibiger HC (1992) Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: a retro- and antero-grade transport and immunohistochemical study. J Comp Neurol 323:387-410.
Stachniak TJ, Ghosh A, Sternson SM (2014) Chemogenetic synaptic silencing of neural circuits localizes a hypothalamus-->midbrain pathway for feeding behavior. Neuron 82:797-808.
Standaert DG, Saper CB, Rye DB, Wainer BH (1986) Colocalization of atriopeptin-like immunoreactivity with choline acetyltransferase- and substance P-like immunoreactivity in the pedunculopontine and laterodorsal tegmental nuclei in the rat. Brain Res 382:163-168.
Swerdlow NR, Geyer MA (1993) Prepulse inhibition of acoustic startle in rats after lesions of the pedunculopontine tegmental nucleus. Behav Neurosci 107:104-117.
Thiel CM, Huston JP, Schwarting RK (1998) Hippocampal acetylcholine and habituation learning. Neuroscience 85:1253-1262.
Typlt M, Mirkowski M, Azzopardi E, Ruth P, Pilz PK, Schmid S (2013) Habituation of reflexive and motivated behavior in mice with deficient BK channel function. Front Integr Neurosci 7:79.
Valsamis B, Schmid S (2011) Habituation and prepulse inhibition of acoustic startle in rodents. J Vis Exp:e3446.
Williams JM, Hamilton LW, Carlton PL (1975) Ontogenetic dissociation of two classes of habituation. J Comp Physiol Psychol 89:733-737.
Wirtshafter D, Stratford TR (2016) Chemogenetic inhibition of cells in the paramedian midbrain tegmentum increases locomotor activity in rats. Brain Res 1632:98-106.
Witten IB, Steinberg EE, Lee SY, Davidson TJ, Zalocusky KA, Brodsky M, Yizhar O, Cho SL, Gong S, Ramakrishnan C, Stuber GD, Tye KM, Janak PH, Deisseroth K (2011) Recombinase-driver rat lines: tools, techniques, and optogenetic application to dopamine-mediated reinforcement. Neuron 72:721-733.

Wynn JK, Dawson ME, Schell AM, McGee M, Salveson D, Green MF (2004) Prepulse facilitation and prepulse inhibition in schizophrenia patients and their unaffected siblings. Biol Psychiatry 55:518-523.

Xiao C, Cho JR, Zhou CY, Treweek JB, Chan K, McKinney SL, Yang B, Gradinaru V (2016) Cholinergic Mesopontine Signals Govern Locomotion and Reward through Dissociable Midbrain Pathways. Neuron 90:333-347.

Yamamoto K, Tanei Z, Hashimoto T, Wakabayashi T, Okuno H, Naka Y, Yizhar O, Fenno LE, Fukayama M, Bito H, Cirrito JR, Holtzman DM, Deisseroth K, Iwatsubo T (2015) Chronic optogenetic activation augments abeta pathology in a mouse model of Alzheimer disease. Cell Rep 11:859-865.

Yeomans J (2012) Muscarinic Receptors in Brain Stem and Mesopontine Cholinergic Arousal Functions. In: Muscarinic Receptors (Fryer AD, Christopoulos, A. & Nathanson, N.M., ed), pp 243-263: Springer.

Yeomans JS, Pollard BA (1993) Amygdala efferents mediating electrically evoked startle-like responses and fear potentiation of acoustic startle. Behav Neurosci 107:596-610.

Yeomans JS, Bosch D, Alves N, Daros A, Ure RJ, Schmid S (2010) GABA receptors and prepulse inhibition of acoustic startle in mice and rats. Eur J Neurosci 31:2053-2061.

Zaman T, Lee K, Park C, Paydar A, Choi JH, Cheong E, Lee CJ, Shin HS (2011) Cav2.3 channels are critical for oscillatory burst discharges in the reticular thalamus and absence epilepsy. Neuron 70:95-108.

Zaman T, Zhou X, Pandey NR, Qin Z, Keyhanian K, Wen K, Courtney RD, Stewart AF, Chen HH (2014) LMO4 is essential for paraventricular hypothalamic neuronal activity and calcium channel expression to prevent hyperphagia. J Neurosci 34:140-148.

5. Chapter 5: General Discussion

5.1 A Summary of Cholinergic Modulations of the Acoustic Startle Response

Appropriate filtering of unnecessary sensory information is critical for normal cognitive functioning. To study sensory filtering, I used prepulse inhibition and habituation of the ASR. I manipulated cholinergic activity using a variety of methods including transgenic and KO mice models, DREADDs and optogenetics. The results are summarized in Table 1.

In Chapter 2, I reported that reduced cholinergic tone did not impact PPI or short-term habituation, but impaired long-term habituation. I was able to rescue this deficit by enhancing remaining cholinergic activity via galantamine. This is the first evidence that ACh is involved in long-term habituation of the ASR, as ACh was previously assumed to have no role in habituation of reflexive behaviours.

Next, narrowing my research focus, I investigated which specific cholinergic receptors might mediate cholinergic modulation of the ASR. To do so I examined habituation and PPI in an α7-nAChR KO mouse line (Chapter 3). Disruptions in this receptor have been documented in schizophrenic populations and have been correlated with other measures of auditory gating (Freedman et al., 1997; Freedman et al., 2000; Leonard et al., 2002). I found normal long- and short-term habituation, but mildly impaired PPI in these mice. This suggests that the α7-nAChR is not critically involved in mediating PPI. Despite impairments in PPI, I found no correlation with higher cognitive spatial abilities in the Barnes maze task. Future studies may want to investigate if sensory filtering and sensorimotor gating disruptions correlate better with differential aspects of cognition (e.g. sustained attention) to provide an overview of how these processes relate to the broad spectrum of cognition (Cyr et al., 2015).

I also found exciting evidence that nicotine-induced enhancement of startle magnitude and PPI were absent in α7-nAChR KO mice. This is the first evidence that narrows the mechanism of this PPI enhancement by nicotine to a single receptor. It also adds another layer to the well documented pro-cognitive effects α7-nAChR agonism, further supporting the targeting of these receptors as treatment options for individuals with PPI impairments, namely schizophrenia (Martin et al., 2004; Olincy et al., 2006).

Lastly, my goal was to pinpoint to what extent midbrain cholinergic neurons in the PPT are responsible for startle modulations using the newly available tools of optogenetics and DREADDs. For the first time, this allowed for the transient and specific activation/inactivation of these neurons during sensory filtering and sensorimotor gating tasks. The PPT has largely been hypothesized to provide inhibitory cholinergic input that mediates PPI (Koch et al., 1993; Swerdlow and Geyer, 1993; Fendt et al., 2001). In contrast to this long-standing concept, I found that inactivation of these neurons reduces startle magnitude and enhances short-term habituation, whereas activation of PPT cholinergic neurons increases startle magnitude. I interpreted these results to suggest that cholinergic PPT neurons modulate startle reactivity, but not in the way the field traditionally thought. It appears that the dominant effect of cholinergic neurons is to enhance, not inhibit, startle. I suggest that this modulation fits well with the general role of these neurons in arousal and discuss that this may also be a mechanism of startle sensitization. In fact, one possibility is that the PPT may serve as a locus of plasticity and integration for long-term modulation of startle (i.e. long-term habituation or sensitization). Fitting with its role in arousal and association of environments and rewards (Olmstead and Franklin, 1993; Kobayashi et al., 2002; Petzold et al., 2015; Xiao et al., 2016), these cells could

serve to tonically regulate startle-mediating PnC excitability. I found no role of these neurons in regulating locomotor activity, adding to the growing body of literature that suggests that PPT neurons are a center for early action selection rather than a locomotor area (Gut & Winn, 2016).

5.1.1 Future Directions

Overall, my studies did not reveal a substantial role for Ach signaling in PPI, contrary to the predominant theories in the field. This may suggest that ACh only modulates, but not mediates, PPI. This is clearly illustrated in Table 1: we used several different manipulations of cholinergic activity, none of which profoundly impacted PPI. In fact, the only impairment of PPI we found was a mild deficit in α7-nAChR KO mice; additionally optogenetic stimulation of cholinergic neurons facilitated, not inhibited, startle response magnitude. Therefore, it is likely that other neurotransmitters play a more important role than traditionally thought.

Overall, my evidence suggests that the neural circuitry of PPI may need a new framework that remedies its current emphasis on the cholinergic midbrain. Firstly, better understanding the independent roles of GABAergic or glutamatergic cells within the PPT is critical as it is probable that one, or both, of these cell types are very important for PPI, since general lesions to the PPT reliably induced PPI deficits (Koch et al 1993; Swerdlow and Geyer, 1993; MacLaren et al., 2014).

Additionally, it would be interesting to investigate other potential sources of cholinergic input to the PnC or other areas within the startle circuitry that may impact PPI. Better understanding of cholinergic inputs to the PnC and other brain regions involved in PPI is the key to determining the site of cholinergic modulation of PPI as pharmacological studies have reliably documented this effect (Fendt and Koch, 1999; Jones and Shannon, 2000a, b; Yeomans et al.,

2010; Pinnock et al., 2015). As our evidence suggests that cholinergic PPT neurons may not be as critical as traditionally thought, these types of future studies will help us more accurately reframe the PPI network (figure 1.3). Future studies should consider examining a recently suggested circuit for PPI that entails cholinergic neurons of the ventral nucleus of the trapezoid body modulating the activity of cochlear root neurons, a critical junction within the primary startle pathway (Gomez-Nieto et al., 2008; Gomez-Nieto et al., 2014, see figure 1.3).

Furthermore, as my results also suggest a new role for the PPT in startle modulation, i.e. maintaining and potentially sensitizing general startle reactivity, this role will need to be further studied (see Table 1). Firstly, I hypothesize that the observed results are mediated mainly by the subset of cholinergic neurons that co-express Substance P. Differentially examining the role of cholinergic neurons that co-express substance P versus those that do not, could uncover a subtle network that may exist within the PPT. Secondly, our observed midbrain cholinergic modulation of startle magnitude may suggest that this center may be the locus of plasticity necessary for long-term habituation. This locus was previously proposed (see Jordan and Leaton, 1983; Leaton et al., 1985), however the field has not yet determined the site for this integration. We believe the cholinergic midbrain is of particular interest because in Chapter 2 we highlighted that ACh was important for long-term habituation. Due to methodological considerations, I was unable to include an investigation of long-term habituation in Chapter 4, so ongoing studies are currently addressing this.

5.2 Differential Cholinergic Modulation of Reflexive vs. Non-Reflexive Behaviours

It has been well documented that ACh differentially modulates habituation of reflexive and non-reflexive behaviours (Hughes, 1984). To examine this issue, I compared the habituation of the ASR compared to locomotor behaviour in α7-nAChR KO mice, as well as during inhibition of cholinergic PPT neurons. In both cases I did not find an impact on habituation of locomotor behaviour. This highlights the potential importance of other cholinergic brain regions, e.g. the hippocampus, in this type of behaviour.

I reported that inhibition of cholinergic PPT neurons improved startle habituation, likely via a reduction in sensitization, without impacting locomotor habituation. This suggests that both habituation and sensitization of reflexive vs. non-reflexive behaviours is differentially mediated. Again, this highlights the complexity of related, but distinct, cognitive processes and shows that there is no ubiquitous sensory filtering mechanism. It is important to identify these subtle distinctions underlying these processes so that we can identify common treatment targets for the shared hallmarks of cognitive dysfunction across diseases.

5.3 The Role of Nicotinic Receptors in Startle Modulation

I have reported that nicotinic receptors, particularly the α7-nAChR modulate PPI (Chapter 3). I have also demonstrated that nicotinic receptors play a key role in the modulation of startle magnitude. Nicotine-induced enhancement of startle magnitude was absent in α7-nAChR mice, and global nicotinic blockade was able to prevent optogenetically-induced enhancement of startle. Future studies should seek to determine at what points within the startle circuitry this

modulation is occurring, and what the physiological or behavioural relevance of startle magnitude may indicate.

Many disorders have been documented to have an increase in startle magnitude including Post-Traumatic Stress Disorder (Grillon et al., 1996; however, see Morgan et al., 1996) or Generalized Anxiety Disorder (Ray et al., 2009). We need to better understand the mechanisms underlying enhancement of startle magnitude, including PPF or sensitization, in order to help find treatment options for populations like this. However, it should be noted that ACh has not been strongly linked to either of these disorders.

Manipulation of Cholinergic Activity	Impact on PPI	Impact on Baseline Startle Magnitude	Impact on STH	Impact on LTH
Global Reduction of Cholinergic Tone (via VAChT KD)	None	None	None	Grossly impaired, rescued by ACh agonism
Global KO of the α7-nAChR	Mild impairment, nicotine-induced enhancement of PPI is absent	None, nicotine-induced enhancement of startle is absent	None	No Impact
Chemogenetic Inhibition of Cholinergic PPT Neurons	None	Reduced	Slight enhancement	N/A
Optogenetic Activation of Cholinergic PPT Neurons	Facilitates startle response: (-%PPI)	Enhanced, blocked by nicotinic antagonism	May induce sensitization	N/A

Table 5.1: A Summary of Results

Overall, manipulations of cholinergic activity did not impact PPI. While we were able to see a mild contribution of nicotinic receptors to PPI, a global knock-down of ACh did not impact PPI.

Furthermore, manipulation of cholinergic neurons in the midbrain nucleus, the PPT, impacted startle response magnitude in the opposite manner traditionally supposed by the field. Our results suggest that ACh plays a minor role in PPI but that future studies should re-examine the role of other neurotransmitters in this process.

5.4 Conclusions

This thesis highlights the modulatory role of cholinergic activity during the processing of the acoustic startle response. My results indicated that cholinergic signaling is not critical for mediating PPI, as traditionally hypothesized. Instead, my studies demonstrate that the most robust effect of cholinergic modulation is to maintain startle reactivity and potentially regulate sensitization of the startle response, which seems to be at least partially mediated through nicotinic receptors. This is summarized in Table 1. Future studies should seek to further understand if this midbrain cholinergic modulation of the ASR is critical for sensitization and its potential role in long-term habituation phenomena. In addition, my findings would suggest that the functional neuronal circuitry underlying PPI needs to be re-evaluated, starting with distinguishing GABAergic vs glutamatergic modulations of PPI within the PPT.

5.5 References

Cyr M, Parent MJ, Mechawar N, Rosa-Neto P, Soucy JP, Clark SD, Aghourian M, Bedard MA (2015) Deficit in sustained attention following selective cholinergic lesion of the pedunculopontine tegmental nucleus in rat, as measured with both post-mortem immunocytochemistry and in vivo PET imaging with [(1)(8)F]fluoroethoxybenzovesamicol. Behav Brain Res 278:107-114.

Fendt M, Koch M (1999) Cholinergic modulation of the acoustic startle response in the caudal pontine reticular nucleus of the rat. Eur J Pharmacol 370:101-107.

Fendt M, Li L, Yeomans JS (2001) Brain stem circuits mediating prepulse inhibition of the startle reflex. Psychopharmacology (Berl) 156:216-224.

Freedman R, Adams CE, Leonard S (2000) The alpha7-nicotinic acetylcholine receptor and the pathology of hippocampal interneurons in schizophrenia. J Chem Neuroanat 20:299-306.

Freedman R et al. (1997) Linkage of a neurophysiological deficit in schizophrenia to a chromosome 15 locus. Proc Natl Acad Sci U S A 94:587-592.

Gomez-Nieto R, Rubio ME, Lopez DE (2008) Cholinergic input from the ventral nucleus of the trapezoid body to cochlear root neurons in rats. J Comp Neurol 506:452-468.

Gomez-Nieto R, Sinex DG, Horta-Junior Jde A, Castellano O, Herrero-Turrion JM, Lopez DE (2014) A fast cholinergic modulation of the primary acoustic startle circuit in rats. Brain Struct Funct 219:1555-1573.

Grillon C, Morgan CA, Southwick SM, Davis M, Charney DS (1996) Baseline startle amplitude and prepulse inhibition in Vietnam veterans with posttraumatic stress disorder. Psychiatry Res 64:169-178.

Hughes RN (1984) Cholinergic and monoaminergic substrates of startle habituation. Life Sci 34:2101-2105.

Jones CK, Shannon HE (2000a) Muscarinic cholinergic modulation of prepulse inhibition of the acoustic startle reflex. J Pharmacol Exp Ther 294:1017-1023.

Jones CK, Shannon HE (2000b) Effects of scopolamine in comparison with apomorphine and phencyclidine on prepulse inhibition in rats. Eur J Pharmacol 391:105-112.

Jordan WP, Leaton RN (1983) Habituation of the acoustic startle response in rats after lesions in the mesencephalic reticular formation or in the inferior colliculus. Behav Neurosci 97:710-724.

Kobayashi Y, Inoue Y, Yamamoto M, Isa T, Aizawa H (2002) Contribution of pedunculopontine tegmental nucleus neurons to performance of visually guided saccade tasks in monkeys. J Neurophysiol 88:715-731.

Koch M, Kungel M, Herbert H (1993) Cholinergic neurons in the pedunculopontine tegmental nucleus are involved in the mediation of prepulse inhibition of the acoustic startle response in the rat. Exp Brain Res 97:71-82.

Leaton RN, Cassella JV, Borszcz GS (1985) Short-term and long-term habituation of the acoustic startle response in chronic decerebrate rats. Behav Neurosci 99:901-912.

Leonard S, Gault J, Hopkins J, Logel J, Vianzon R, Short M, Drebing C, Berger R, Venn D, Sirota P, Zerbe G, Olincy A, Ross RG, Adler LE, Freedman R (2002) Association of promoter

variants in the alpha7 nicotinic acetylcholine receptor subunit gene with an inhibitory deficit found in schizophrenia. Arch Gen Psychiatry 59:1085-1096.
Martin LF, Kem WR, Freedman R (2004) Alpha-7 nicotinic receptor agonists: potential new candidates for the treatment of schizophrenia. Psychopharmacology (Berl) 174:54-64.
Morgan CA, 3rd, Grillon C, Southwick SM, Davis M, Charney DS (1996) Exaggerated acoustic startle reflex in Gulf War veterans with posttraumatic stress disorder. Am J Psychiatry 153:64-68.
Olincy A, Harris JG, Johnson LL, Pender V, Kongs S, Allensworth D, Ellis J, Zerbe GO, Leonard S, Stevens KE, Stevens JO, Martin L, Adler LE, Soti F, Kem WR, Freedman R (2006) Proof-of-concept trial of an alpha7 nicotinic agonist in schizophrenia. Arch Gen Psychiatry 63:630-638.
Olmstead MC, Franklin KB (1993) Effects of pedunculopontine tegmental nucleus lesions on morphine-induced conditioned place preference and analgesia in the formalin test. Neuroscience 57:411-418.
Petzold A, Valencia M, Pal B, Mena-Segovia J (2015) Decoding brain state transitions in the pedunculopontine nucleus: cooperative phasic and tonic mechanisms. Front Neural Circuits 9:68.
Pinnock F, Bosch D, Brown T, Simons N, Yeomans JR, DeOliveira C, Schmid S (2015) Nicotine receptors mediating sensorimotor gating and its enhancement by systemic nicotine. Front Behav Neurosci 9:30.
Ray WJ, Molnar C, Aikins D, Yamasaki A, Newman MG, Castonguay L, Borkovec TD (2009) Startle response in generalized anxiety disorder. Depress Anxiety 26:147-154.
Swerdlow NR, Geyer MA (1993) Prepulse inhibition of acoustic startle in rats after lesions of the pedunculopontine tegmental nucleus. Behav Neurosci 107:104-117.
Xiao C, Cho JR, Zhou CY, Treweek JB, Chan K, McKinney SL, Yang B, Gradinaru V (2016) Cholinergic Mesopontine Signals Govern Locomotion and Reward through Dissociable Midbrain Pathways. Neuron 90:333-347.
Yeomans JS, Bosch D, Alves N, Daros A, Ure RJ, Schmid S (2010) GABA receptors and prepulse inhibition of acoustic startle in mice and rats. Eur J Neurosci 31:2053-2061.

A. Appendix A

Contained in this Appendix are the supplementary figures for Chapter 4

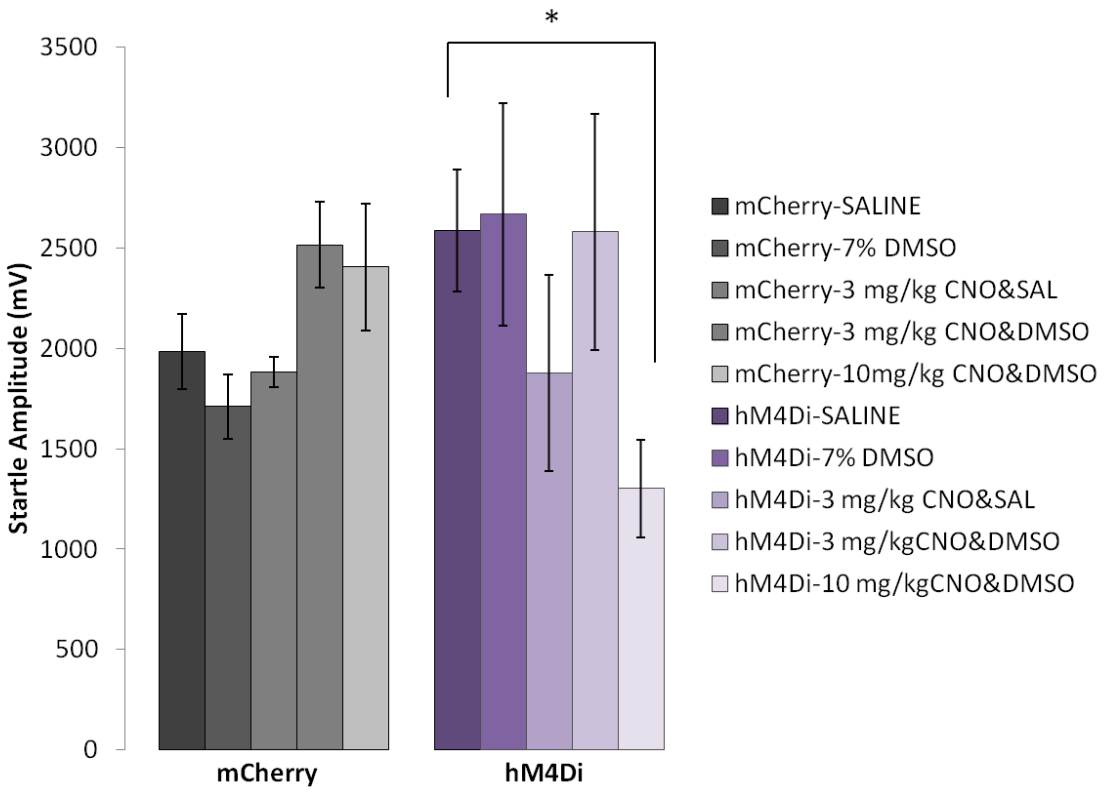

Figure A.1 Pilot Data Determining Effective CNO Dosage

Pilot testing demonstrated that only at a 10 mg/kg dose of CNO we were able to see a differential effect in hM4Di animals compared to mCherry controls. At this dose, hM4Di animals show significantly decreased startle amplitudes (raw data plotted, t_7=3.6, p=0.004). This was not apparent in control animals, or at any other dose tested (3 mg/kg in saline or 3 mg/kg in DMSO). Therefore, this dose was selected for future experiments (n=4/group). Animals also completed habituation and PPI testing. No effect of CNO was observed (data not shown).

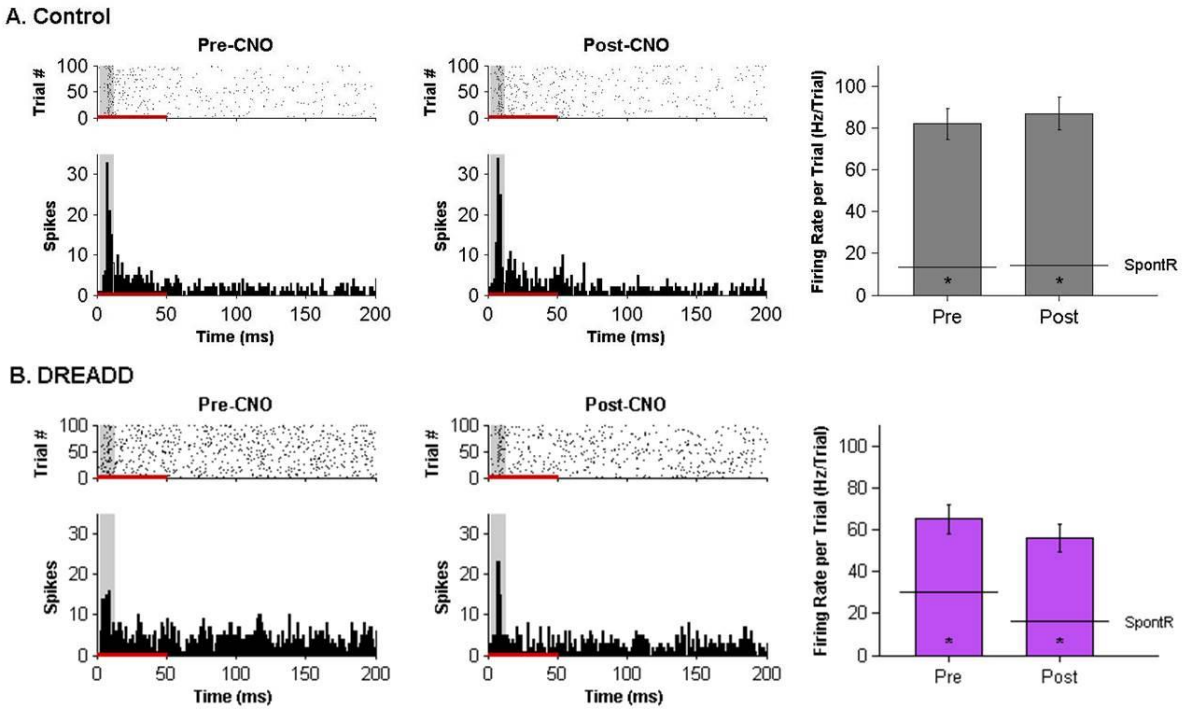

Figure A.2 Representative *In Vivo* Electrophysiological Spiking Activity in hM4Di and Control Animals

A) Representative rastor plots (top row) and PSTH (bottom row) of evoked activity to an 85 dB SPL noise burst in the PPT of an example mCherry control animal or B) hM4Di expressing animal before and after systemic CNO administration. The average firing rate for each trial was calculated using the spiking activity during 2-12 ms from stimulus onset. This is plotted in the right bar graphs for both example animals. Stimulus onset and duration is noted by the red line on rastor and PSTHs. The average spontaneous activity rate was defined by using the last 100 ms of each trial. This is shown by the line on the bar graphs labelled SpontR. Most notably, these examples recapitulate that in hM4Di animals spontaneous activity was reduced by CNO but auditory evoked activity was not. No changes were seen in control mCherry animals.

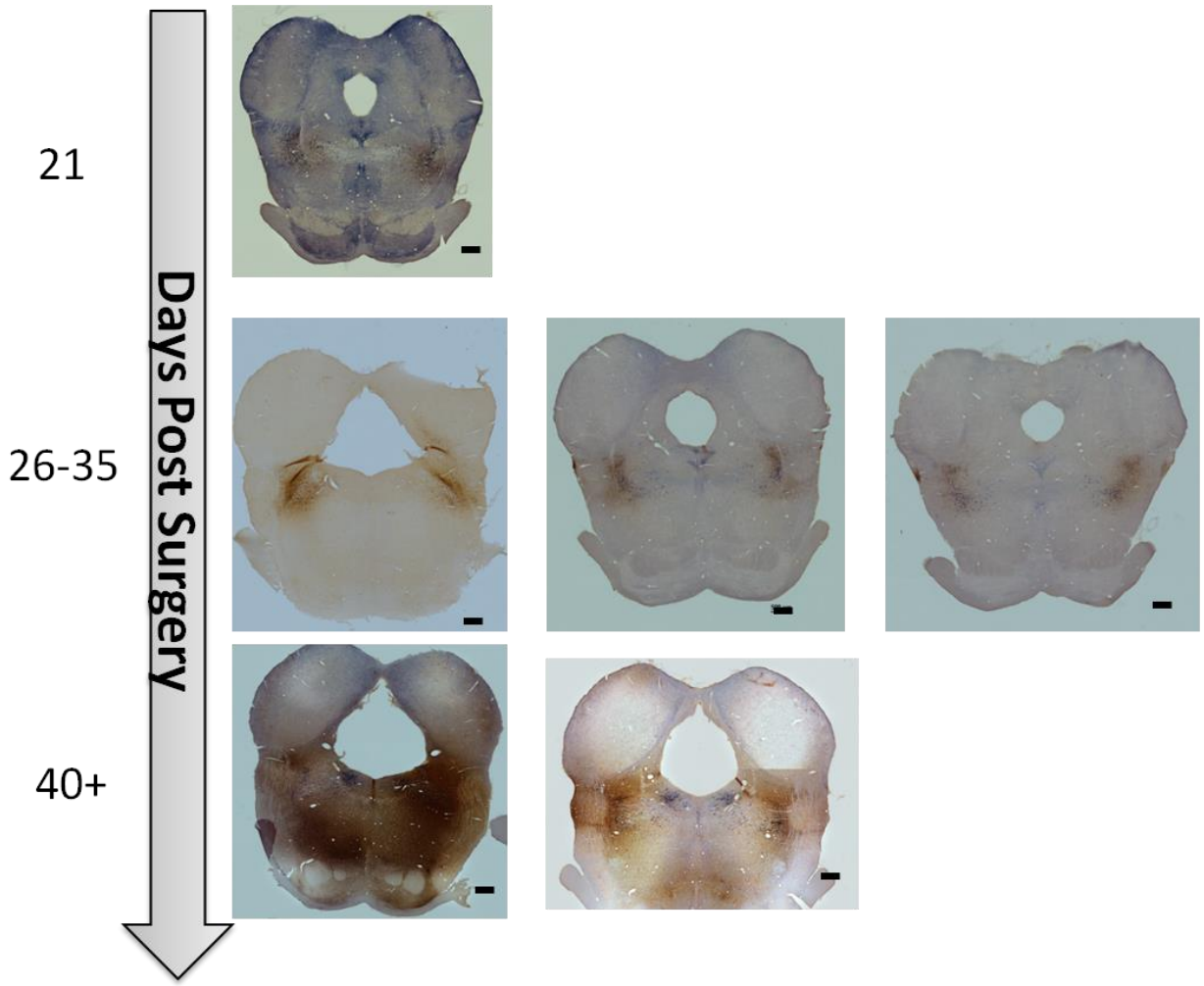

Figure A.3 mCherry Expression Changes with Time

As time progressed, mCherry expression changed. Shown in brown is mCherry staining (DAB) and NADPH staining of cholinergic neurons (blue). The brown appeared to intensify with time progression beyond 40 days after surgery. Please note that this was not due to an increase the number of labelled neurons, but rather diffuse staining of the neuropil. This could be due to improved mCherry trafficking to neuronal processes, or an indicator of fluorescent protein accumulative toxicity. All behavioural and electrophysiological experiments were performed between days 21-35. Images were taken at 2x magnification, scale bar is 500 µM.

Startle Facilitation is Frequency Dependent

In order to determine the most effective photostimulation paradigm, we ran a subset of animals with 10 and 1 Hz stimulation (YFP n=3, ChR2 n=4) in addition to the previously discussed 50 Hz (YFP n=6, ChR2 n=7). We analysed this using a three-way repeated measures ANOVA (virus × frequency × ISI). Again, we confirmed that only ChR2 expressing animals showed a facilitation of startle as there was a main effect of virus type($F_{(1,20)}$=14, p<0.001). While we observed a main effect of stimulation frequency ($F_{(1,20)}$=8.8, p<0.01), most importantly we also found a significant interaction between virus type and frequency ($F_{(1,20)}$=8.7, p<0.01). This denotes that some photostimulation frequencies were more effective than others in the ChR2 expressing animals. Post-hoc t-tests with Bonferroni corrections revealed that in ChR2 animals, the 50 Hz stimulation was significantly different than the 10 and 1 Hz paradigms (independent samples t-test: t_{45}=5.0, p>0.001, t_{45}=3.5, p>0.005 respectively). This highlights 50 Hz photostimulation as the most effective, as shown in figure A.4A

Startle Facilitation is Maximal with Bilateral (vs. Unilateral) Photostimulation

In a subset of ChR2 expressing animals (n=3), we stimulated at 50 Hz unilaterally in order to see whether there are any lateralization affects. A two way repeated measures ANOVA (stimulation laterality × ISI) found no statistical difference between unilateral and bilateral stimulation on PPI ($F_{(1,2)}$=3.2, p>0.05) or interaction between interstimulus interval and stimulation ($F_{(3,6)}$=1.7, p>0.05). However, it appeared that within animals unilateral stimulation produced roughly half the increase of bilateral stimulation (figure A.4B).

Startle Facilitation is Light-Intensity Dependant

We documented that the facilitation of startle was also dependant on the intensity of LED photostimulation. We ran a subset of ChR2 expressing animals (n=3) with high (19.6-22.1 mW) or low intensity (1-4 mW) photostimulation. We analysed this using a two-way repeated measures ANOVA (photostimulation intensity × ISI). Low intensity photostimulation did not enhance startle as effectively as we found a main effect of intensity ($F_{(1,2)}$=24.8, p<0.05). In this group, startle magnitude was always greater in the high intensity stimulation condition as there was no interaction between intensity and interstimulus interval ($F_{(3,6)}$=1.6, p>0.05). Overall this indicated that sufficient light intensity, not just presentation of light alone, was necessary to facilitate startle, as demonstrated in figure A.4C.

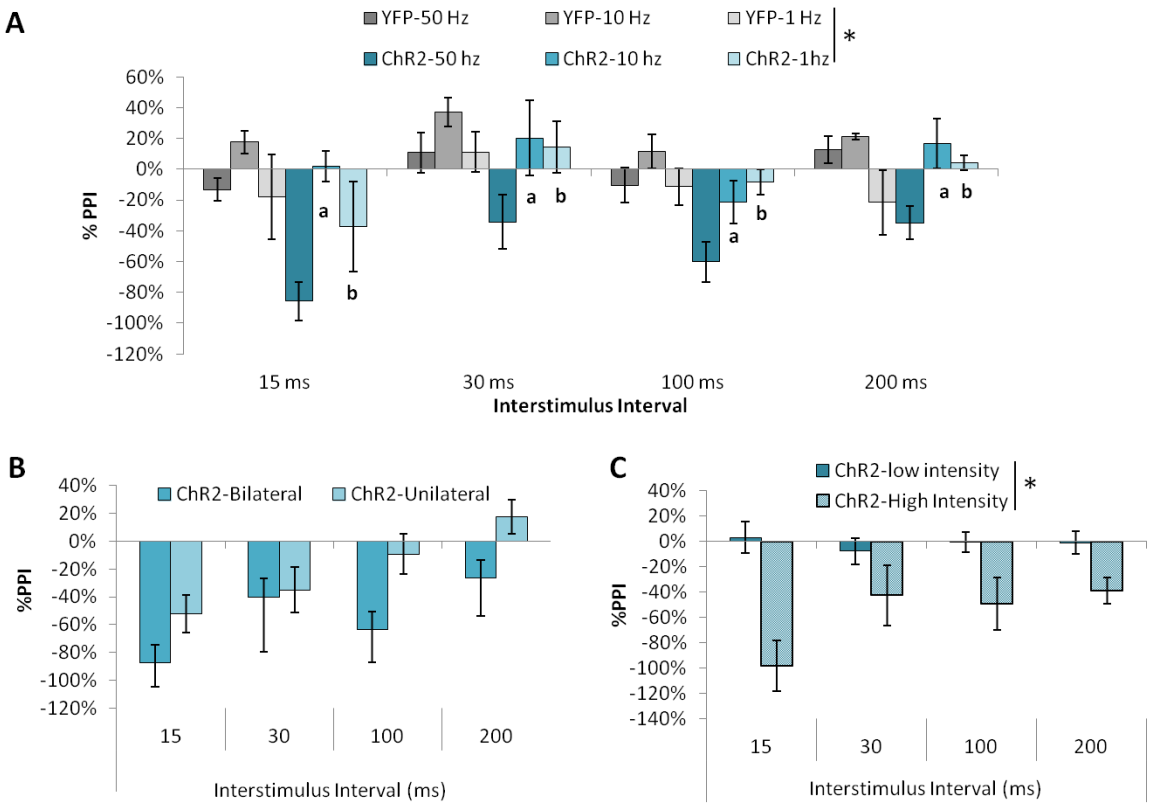

Figure A.4 Facilitation of Startle is Dependant on Frequency, Laterality and Light Intensity

As shown in A) we reconfirmed that photostimulation was only effective in ChR2 expressing animals, as denoted by the asterisk. Furthermore, we observed that stimulation frequency differentially altered startle magnitude. We found that 50 Hz stimulation produces significantly more enhancement of startle compared to 10 (denoted by a) or 1 Hz (denoted by b), across all ISIs (50 Hz YFP n=6, ChR2=7, 10 and 1 Hz YFP n=3, ChR2=4). B) In a subset of animals (n=3), unilateral (compared to bilateral) stimulation of the PPT produced a similar, but not quite as large, enhancement of startle. However, this trend failed to reach statistical significance. Lastly, as shown in C) this enhancement of startle was dependant on the intensity of stimulation. Low intensity photostimulation (1-4 MW) failed to increase startle across all ISIs intervals, unlike high intensity stimulation (n=3).

B. Appendix B

Western

AUP Number: 2008-010-03
PI Name: Schmid, Susanne
AUP Title: Mechanisms Underlying Habituation And Prepulse Inhibition Of Startle

Approval Date: 04/20/2016

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Mechanisms Underlying Habituation And Prepulse Inhibition Of Startle

" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2008-010-03::9

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

C. Appendix C

This Agreement between Erin C Azzopardi ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	3906571478183
License date	Jul 12, 2016
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Genes, Brain and Behavior
Licensed Content Title	VAChT knock-down mice show normal prepulse inhibition but disrupted long-term habituation
Licensed Content Author	S. Schmid,E. Azzopardi,X. De Jaeger,M. A. M. Prado,V. F. Prado
Licensed Content Date	Mar 15, 2011
Licensed Content Pages	8
Type of Use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Understanding the Role of Cholinergic Modulation of Sensory Filtering and Sensorimotor Gating Mechanisms

This Agreement between Erin C Azzopardi ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	3906571478183
License date	Jul 12, 2016
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Genes, Brain and Behavior
Licensed Content Title	VAChT knock-down mice show normal prepulse inhibition but disrupted long-term habituation
Licensed Content Author	S. Schmid,E. Azzopardi,X. De Jaeger,M. A. M. Prado,V. F. Prado
Licensed Content Date	Mar 15, 2011
Licensed Content Pages	8
Type of Use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Understanding the Role of Cholinergic Modulation of Sensory Filtering and Sensorimotor Gating Mechanisms

D. Appendix D

Erin Azzopardi

EDUCATION

University of Western Ontario
PhD in Anatomy and Cell Biology **09/2010-current**

Wilfrid Laurier University
BSc Honours in Psychology, Research Specialty **09/2005-05/2010**
Minor: Biology
Honours Thesis: Consolidation-Related Gene Expression is Preserved in the Aged Dentate Gyrus

SCHOLARSHIPS

Alexander Graham Bell Canada Graduate Doctoral Scholarship (CGSD2), University of Western Ontario **2014-2016**
Western Graduate Research Scholarship, University of Western Ontario **2010-2016**
Ontario Graduate Scholarship, Declined **2014**
Ontario Graduate Scholarship, University of Western Ontario **2013-2014**
Ontario Graduate Scholarship, University of Western Ontario **2012-2013**
Deans MSc to PhD Transfer Scholarship, Declined **2012-2013**
In-course Scholarship Level 2, Wilfrid Laurier University **2009-2010**
In-course Scholarship Level 2, Wilfrid Laurier **2008-2009**
Entrance Scholarship, Wilfrid Laurier University **2005-2006**

AWARDS

Southern Ontario Neuroscience Association Chapter Travel Award **2014**
Schulich Scholarship for Medical Research **2014**
Graduate Thesis Research Award, University of Western Ontario **January 2012**

TEACHING EXPERIENCE

Head Teaching Assistant
Integrative Neuroscience **2014**

Guest Lecturer
Histology ACB3319 **2014**

Teaching Assistant
Integrative Neuroscience **2010-2013**

Tutor Doctor, London Ontario
Tutor-Grade 11 Mathematics **2012-2013**

Learn to Learn Differently, London Ontario
Volunteer Tutor- Junior Kindergarten **2010-2011**

Students Offering Support, Wilfrid Laurier University
Volunteer Tutor-Grade 11 Biology **2009-2010**

RELATED EXPERIENCE

Policy Committee, Society of Graduate Students, University of Western Ontario
Member **2014-2015**

Space Committee, Schulich School of Medicine and Dentistry, University of Western Ontario
Member, Graduate Student Representative **2014-2015**

Anatomy and Cell Biology, University of Western Ontario
Elected Co-Chair of Anatomy and Cell Biology Student Council **2012-2014**

Graduate Affairs Committee, Anatomy and Cell Biology, University of Western Ontario
Member, Graduate Student Representative **2012-2014**

Society of Graduate Students, University of Western Ontario
Anatomy and Cell Biology Graduate Student Representative **2011-2012**

Advisory Committee Member (Undergraduate Thesis)

Training Course
Dr. K. Diesseroth's Optogenetic Innovation Laboratory (OIL), Stanford University **2013**

Anatomy and Cell Biology, University of Western Ontario
Elected Research Society of Graduate Student Representative **2011-2012**

Dale Brain Injury Services, London Ontario
Leisure Volunteer Visitor **2012-2013**

Brandon Hall Research
Research Assistant
January 2010

Marrone Lab, Wilfrid Laurier University
Research Assistant **January 2008 – January 2009**

PUBLICATIONS AND PAPERS

Azzopardi, E., Typlt, M., Jenkins, B. & Schmid, S. (2013). Sensory filtering and spatial learning in α7 nicotine receptor knock-out mice. Genes, Brain and Behaviour. 12:414-423.

Azzopardi, E., Shaikh, K., Typlt M., Mirkowski, M., Barros, W. & Schmid, S. Effective Measure of the Acoustic Startle Response in Rodents. (submitted to Frontiers in Intergrative Neuroscience).

Gheidi, A., Azzopardi, E., Adams, A.A. & Marrone, D.F. (2013). Experience-dependent persistent expression of zif268 during rest is preserved in the aged dentate gyrus. BMC Neuroscience 2013, 14:100.

Schmid, S., Azzopardi, E., De Jaeger, Prado, M.A.M. & Prado, V.F. (2010). VAChT Knock-down mice show normal prepulse inhibition but disrupted long-term habituation. Genes, Brain and Behaviour. 10:457-464.

Typlt, M., Mirkowski, M., Azzopardi, E., Ruettiger, L., Ruth, P. & Schmid, S. (2013). Mice with deficient BK channel function show impaired prepulse inhibition and spatial learning, but normal working and spatial reference memory. PLoS One, 8:e81270.

Typlt, M., Mirkowski, M., Azzopardi, E., Ruth, P., Pilz, P.K.D. & Schmid, S. (2013). Habituation of reflexive and motivated behaviour in mice with a deficiency of the α-subunit of the BK channel. Frontiers in Integrative Neuroscience, 7:79.

CONFERENCES & PRESENTATIONS

Azzopardi, E., Schmid, S. Investigating cholinergic function of the pedunculopontine tegmental nucleus in sensorimotor filtering. Society for Neuroscience (SFN) 2014, Washington DC.

Schmid, S., Azzopardi, E., Typlt, M., Robinson, J., Mirkowski, M. & Pilz, P. Mechanisms underlying short-term versus long-term habituation of evoked vs. motivated behaviour. European Brain and Behaviour Society Meeting 2013, Munich GER. Poster Presentation.

Azzopardi E., Typlt M. & Schmid, S. α7 nicotinic receptor knock-out mice show impaired prepulse inhibition and cognitive alterations. Southern Ontario Neuroscience Association (SONA) 2012 Toronto ON, Society of Neuroscience (SFN) 2012 New Orleans LA. Canadian Society of Neuroscience (CAN), 2012, Toronto ON. Poster Presentation.

Mirkowski, M., Typlt, M., Azzopardi, E., Ruth, P. & Schmid, S. The role of BK potassium channels in habituation and prepulse inhibition of startle. SONA 2012 Toronto ON, SFN 2012 New Orleans LA. CAN 2013, Toronto ON. Poster Presentation.

Azzopardi, E. (2011). Understanding Cholinergic Modulation of Sensory Filtering Mechanisms. Anatomy and Cell Biology Research Day. Western University. Oral Presentation.

Azzopardi,E., Schmid, S., X. De Jaeger, V.F. Prado & M.A.M. Prado. VAChT knock-down mice show normal prepulse inhibition but disrupted long-term habituation. SONA, Guelph ON, (2011) Poster Presentation.

MEMBERSHIPS

Society for Neuroscience
International Behavioral Neuroscience Society
Canadian Association for Neuroscience